The role of immune inhibitory receptors in age-associated immune decline

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I, Richard Macaulay confirm that the work presented in this thesis is my own. Where information has been derived from other sources I confirm that this has been indicated in the thesis.

Signed:

Abstract

The balance between signals delivered by positive and negative costimulatory molecules is crucial to the ultimate fate of cellular immune responses. Manipulation of T cell costimulatory pathways may offer a novel approach for reinvigorating exhausted T cell responses, especially in the context of chronic infections. T cells also display profound exhaustion in old age and this thesis investigates the hypothesis that T cell inhibitory receptor upregulation may define a reversible defect in age onset immune decline.

Data presented here illustrate how T cells utilise different inhibitory receptors as they differentiate and that KLRG1 signalling is causative of dysfunctions in highly differentiated $CD8^+$ T cells. The inhibitory receptors KLRG1 and CTLA-4 are revealed to undergo age-associated upregulations on $CD8^+$ T cells but their blockade does not reverse the characteristic hypo-responsiveness of $CD8^+$ T cells amongst old donors.

The dysregulated immune response to lifelong chronic cytomegalovirus (CMV) infection is thought to play a major role in driving age related immune dysfunctions. We found that CMV accelerates age-associated telomere attrition amongst $CD8^+$ and $CD4^+$ T cells. CMV infection is also shown to drive CTLA-4, PD-1 and KLRG1 upregulation on both $CD4^+$ and $CD8^+$ T cells. Moreover, the PD-1/Ligand (L) inhibitory pathway defines a reversible proliferative dysfunction in the responses of CMV specific $CD8^+$ T cells. Specifically, the CD45RA re-expressing memory subset

exhibits a proliferative deficiency, relative to their central and effector memory counterparts, that is reversible upon PD-L blockade. However, this augmented proliferative response was not accompanied by increased telomerase function, suggesting this does not result in true reversal of exhaustion.

In summary, the dysfunctions of highly differentiated and CMV specific $CD8^+$ T cells can be at least partially reversed by perturbation of inhibitory receptor pathways, whose further manipulation may provide a therapeutic modality to combat age-associated immune decline.

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Publication, Abstract and Poster List

Data I have generated has contributed to the following publications, abstracts and posters: -

Publications

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Henson,S.M., Franzese,O., **Macaulay,R**., Libri,V., Azevedo,R.I., Kiani-Alikhan,S., Plunkett,F.J., Masters,J.E., Jackson,S., Griffiths,S., Pircher,H.P., Soares,M.V. and Akbar,A.N. (2009). KLRG1 signaling induces defective Akt (Ser473) phosphorylation and proliferative dysfunction of highly differentiated CD8+ T cells. Blood.*113(26),* 6619-28.

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Abstracts

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Posters

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List of Abbreviations

AICD	Activation induced cell death
AIDS	Acquired immune deficiency syndrome
APC	Antigen presenting cell
BCR	B cell receptor
bpy	Base pairs per year
cpm	Counts per minute
CMV	Cytomegalovirus
CMV^-	Cytomegalovirus sero-negative
CMV^+	Cytomegalovirus sero-positive
CTLA-4	Cytotoxic T-Lymphocyte Antigen 4
DKC	Dyskeratosis congenita
DDR	DNA damage response
DNA	Deoxyribonucleic acid
EBV	Epstein Barr virus
FCS	Foetal calf serum
FSC	Forward scatter
Flow-FISH	Flow cytometric detection of fluorescence in situ hybridization
HBV	Hepatitis B virus
HCV	Hepatitis C virus
HIV	Human immunodeficiency virus
HSV	Herpes simplex virus
IDO	Indoleamine 2,3 dioxygenase

IE	Immediate early
IFN	Interferon
IL	Interleukin
IRP	Immune risk phenotype
ITAM	Immuno-tyrosine based activation motifs
ITIM	Immuno-tyrosine based inhibitory motif
iTreg cell	Inducible regulatory T cell
kB	kilobase
KLRG1	Killer cell lectin-like receptor subfamily G member 1
L	Ligand
LAG-3	Lymphocyte-activation gene 3
LC	Langerhans cell
LCMV	Lymphocytic chorio-meningitis virus
MFI	Mean fluorescence intensity
MHC	Major histocompatibility complex
NK cell	Natural killer cell
NKR	Natural killer cell receptor
nTreg cell	Naturally occurring regulatory T cell
ORFs	Open reading frames
pAPC	Professional antigen presenting cell
PD-1	Programmed death 1
PD-L	Programmed death ligands
PLC-γ	Phospholipase -C γ
RNA	Ribonucleic acid

SARS	Severe acute respiratory syndrome
SES	Socio-economic status
siRNA	Small interfering RNA
SIV	Simian immunodeficiency virus
SSC	Side scatter
T_{CM}	Central memory T cell
TCR	T cell receptor
T_{EM}	Effector memory T cell
Th1	T helper type 1
Th2	T helper type 2
Tim-3	T-cell immunoglobulin domain and mucin domain 3
T_N	Naïve T cell
TNF	Tumour necrosis factor
Treg cell	Regulatory T cell
T_{REV}	CD45RA revertant memory T cell
VZV	Varicella zoster virus
WT	Wild type
$^{-/-}$	Knockout

1 Introduction

The dramatic expansion of life expectancy in developed countries over the twentieth century, increasing at the rate of several hours per day (Kirkwood, 2008), ranks as one of humanity's great triumphs. However, this achievement, combined with declining birth rates, now form one of society's greatest challenges: the rapid ageing of the world's population. Indeed, by 2031 over a quarter of people worldwide will be over 65 (Khaw, 1999; O'Connell and Ostaszkiewicz, 2005; Rajan et al., 2003; Goldacre, 2009) and the declining health status, economic productivity and cost of care of the aged (McNamee et al., 2001; Robertson and Tracy, 1998) make understanding the mechanisms governing physiological ageing one of the most pertinent challenges facing scientists today.

1.1 How and why we age

1.1.1 How we age

The ageing process is driven by the lifelong accumulation of a wide variety of cellular and molecular damage that leads to age related frailty, disease and ultimately mortality (Kirkwood, 2005). The Mitochondrial Free Radical Theory of Ageing (Harman, 1956) proposes a single physiological cause as the root source of this damage, but is being increasingly refuted (Bonawitz and Shadel, 2007; Buffenstein et al., 2008; Gems and Doonan, 2009; de Magalhaes and Church, 2006; Blagosklonny, 2008; Fukui and Moraes, 2008; Lapointe and Hekimi, 2010). Indeed, ageing is increasingly being recognised as a process with no single cause but being driven by a multitude of factors, including oxidative stress, translation errors, nutritional

components and telomere attrition (Rattan, 2006; Kirkwood, 2008; Hayflick, 2007; Holliday, 2006).

1.1.2 Why we age

Fundamentally, age related changes can occur either as a consequence of a genetically driven program or the stochastic accumulation of damage with age. Programmed death theories proposed that ageing was advantageous to the species by removing aged members from the population (Weismann, 1889). However, traditional Darwinian mechanics exclude the possibility of evolving and retaining a design that produces a net reduction in individual benefit. Moreover, due to high extrinsic mortality in the wild, virtually all animals die before they reach old age, which lead to the formulation of the Theory of Mutation Accumulation, which hypothesised that there is little selection pressure against late acting deleterious traits that cause ageing (Medawar, 1952). This was further developed into the antagonistic pleiotropy hypothesis that proposed natural selection selects genes that are beneficial at early ages but become harmful with advancing age (Williams, 1957). Nevertheless, programmed death theories have enjoyed a resurgence of popularity following the discovery of ageing genes (Ghazi et al., 2007; Francia et al., 2004) and the arisal of alternative ideas of evolutionary mechanics, particularly group selection (Mitteldorf and Pepper, 2007) and evolvability theories (Goldsmith, 2008).

1.2 Immunosenescence

1.2.1 Introduction

1.2.1.1 The significance of age related immune changes

Ageing is accompanied by a progressive, multidimensional, physiological degeneration, with immune system alterations playing a key role regulating these age-related declines (Gorczynski and Terzioglu, 2008).

1.2.1.2 The immune system

Humans are continually exposed to pathogens that attempt to invade and colonise the host. Primary resistance is based on physical barriers but when these are breached the infectious agent encounters a secondary highly organised system of defence called the immune system. Indeed, such pathogenic microorganisms that breach host barriers only occasionally cause disease. The majority are eliminated rapidly, within hours or even minutes, by the mechanisms of innate immunity. If an infectious organism breaches the innate lines of defence then an adaptive immune response ensues. Unlike innate responses, adaptive immunity requires several days for optimal generation but is specific and results in long lived protective immunity. This generation of immunological memory is considered a hallmark of the adaptive immune system and enables rapid and efficient resolution of infection upon pathogen re-encounter (Zinkernagel et al., 1996).

1.2.1.3 The ubiquitous nature of immunosenescence

This age-associated immune deterioration, termed immunosenescence, is ubiquitous and has been documented in a wide range of captive and free-living species including primates (Jayashankar et al., 2003) cats and dogs (Day, 2010), horses (Horohov et al., 2010), birds (Lavoie et al., 2007; Holmes and Austad, 2004; Cichon et al., 2003;

Palacios et al., 2007) and even invertebrates, which lack an adaptive immune system (Adamo et al., 2001; Kurtz, 2002). Amongst such different species, immunosenescence appears as a function of their age relative to their life expectancy rather than chronological time (Ginaldi et al., 2001) and is considered a major contributory factor towards the increase in morbidity and mortality associated with advanced age.

1.2.1.4 Consequences of this age related immune decline

The age related decline in immune function is reflected in infectious diseases being an increasingly important cause of morbidity and mortality amongst the aged, who experience greater infection frequency, duration and severity (High, 2004). This age related immune deficit is particularly noticeable against novel antigenic challenges from previously unencountered pathogens. This is most clearly illustrated by the example of seasonal influenza, which is considered a relatively innocuous disease amongst the young but constitutes a major cause of death amongst the aged (Minino et al., 2002; Simonsen et al., 2007). Furthermore, newly emerging pathogens cause disproportionate amounts of disease and death amongst the aged. Indeed, SARS (Severe Acute Respiratory Syndrome) was rarely fatal in the young but infected elderly experienced mortality rates of over 50% (Donnelly et al., 2003). Additionally, West Nile Virus is mostly asymptomatic amongst immunocompetent hosts but can cause a life threatening neuroinvasive disease that is 40-50 times more common amongst the over 70s compared with those under 40 (Brien et al., 2009). Moreover, many vaccines exhibit reduced efficacy in aged individuals, limiting preventative

prophylaxis (Grubeck-Loebenstein, 2010), being only 17-53% efficacious amongst the aged compared with rates of 70-90% in the young (Goodwin et al., 2006).

Although memory generated from aged individuals functions poorly, memory responses generated towards pathogens encountered before the onset of immunosenescence are well-maintained into old age (Haynes et al., 2003). Indeed, neutralising antibody was recently identified from aged survivors of the 1918 influenza epidemic and in those infected or vaccinated with smallpox as children, despite not being re-exposed to antigen for up to 90 years (Yu et al., 2008; Taub et al., 2008).

1.2.2 How individual immune system components are affected by ageing

Age-associated deleterious changes have been best characterised amongst the adaptive immune system, particularly amongst T cells, which exhibit profound age-associated alterations. Indeed, the diminution of the naïve $CD8^+$ T cell pool with the corresponding reduction in T cell receptor (TCR) diversity and increase in memory cells are seen as hallmarks of immune system ageing (Pawelec et al., 2010c). Although B cell age-associated alterations have been documented, these may largely be a consequence of dysfunctional $CD4^+$ T cell help (Maue and Haynes, 2009). With regard to innate immunity, some reviewers state that its functionality amongst the aged is largely preserved or even enhanced (Franceschi et al., 2000a). Nevertheless, it should be noted that non-immune age related changes also contribute towards immunosenescence, for example the decreased function of epithelial barriers of the

skin, lungs and gastro-intestinal tract amongst the aged permit increased invasion of mucosal tissues by pathogenic organisms (Weiskopf et al., 2009; Bodineau et al., 2009).

1.2.3 T cells

1.2.3.1 Introduction

1.2.3.1.1 Activation of Naïve T cells

T cell progenitors are produced in the bone marrow and mature in the thymus into $CD4^+$ or $CD8^+$ T cells, (further described in section 1.3.1.1). T cells exiting the thymus migrate throughout the body and gather in secondary lymphoid organs. Here they are considered immunologically naïve until they have encountered their specific antigen, which they recognise through their T cell receptor (TCR). TCRs are transmembrane heterodimers consisting of 2 polypeptide chains - predominantly αβ (though a minority of T cells ~5% express a γδ TCR). TCRs possess a conserved segment that participates in the signalling function and a highly variable region responsible for antigen recognition. These variable regions are generated by the stochastic rearrangement of the relevant genes producing a diverse TCR repertoire that can recognise an enormous range of peptides. TCRs recognise antigen in the context of major histocompatibility complex (MHC) molecules that are expressed on the surface of target cells (Zinkernagel and Doherty, 1997). This also requires the binding of a CD4 or CD8 coreceptor that associates with the TCR and binds a conserved region on a MHC class II or class I molecule, respectively. However, a primary immune response can only develop if naïve T cells receive coordinated

stimulation by 3 signals: (1) a specific peptide-MHC complex with high affinity (2) appropriate costimulation (further discussed in section 1.5.1) and (3) inflammatory cytokine signals (Mescher et al., 2006), which result in rapid T cell proliferation accompanied by extensive cellular changes, as T cells acquire effector functions, change their cytokine and chemokine expression and move towards peripheral infection sites as activated effector T cells.

1.2.3.1.2 Effector T cell functions

Naïve $CD8^+$ T cells differentiate into cytotoxic effectors that play critical roles in cellular immune responses directed against intracellular pathogens whereas $CD4^+$ T cells are robust producers of effector cytokines that functionally organise the immune response and provide help to B cell and $CD8^+$ T cell responses (Zhang et al., 2009). Indeed, functionally distinct $CD4^+$ T cell effector subsets exist, whose differentiation decision is determined primarily by the cytokine milieu in the microenvironment (signal 3) (Zhou et al., 2009). T helper type (Th) 1 cells play a key role in cellular immunity, Th2 are required for humoural immunity against extracellular pathogens, Th17 are important in the clearance of extracellular bacteria and fungi particularly at mucosal surfaces, whereas T follicular helpers (Tfh) regulate the maturation of B cell responses (Zhou et al., 2009). Regulatory $CD4^+$ T cell (Tregs) subsets also exist: the thymically derived naturally occurring Tregs (nTregs) and peripherally produced inducible Tregs (iTregs) who dampen effector responses and play critical roles in maintaining peripheral tolerance (Curotto de Lafaille and Lafaille, 2009; Josefowicz and Rudensky, 2009).

1.2.3.1.3 Memory formation

Following successful elimination of the pathogen, a rapid T cell contraction phase ensues resulting in the death of most (90-95%) effector cells with the remaining cells representing memory precursors. This not only increases the number of antigen specific cells in the peripheral T cell pool, as in addition, memory cells differ in function from naïve cells, having a higher antigen affinity, lower activation threshold, being less dependent on costimulation, being able to more rapidly expand and induce effector functions and having altered chemokine and adhesion receptor profiles that enables memory cells to scan peripheral tissues (Rogers et al., 2000). A key feature of memory cells that distinguishes them from effector cells is their ability to persist long term in an antigen independent manner (Surh and Sprent, 2008). Nevertheless, the memory responses to chronic infections differ from the model presented above (further discussed in section 1.4.3).

1.2.3.1.4 Central and effector memory

Memory lymphocytes confer immediate protective immunity in the periphery and can mount proliferative recall responses in secondary lymphoid organs. Amongst B lymphocytes, distinct cell types mediate these functions, with plasma cells secreting antibodies that confer immediate protective immunity and memory B cells mediating reactive memory by proliferating and differentiating to plasma cells in response to antigenic challenge (Lanzavecchia and Sallusto, 2009). Similarly, amongst T cells, protective memory has been suggested to be mediated by effector memory T cells (T_{EM}) that circulate in the periphery and execute immediate effector functions but exhibit limited proliferative responses, whereas central memory cells (T_{CM}) confer

reactive memory, expressing lymphoid homing receptors, exhibiting vigorous proliferation but little immediate effector functions upon secondary antigenic challenge and efficiently differentiating into effector cells (Sallusto et al., 2004).

1.2.3.1.5 CD45RA-revertant memory T cells

An age-associated accumulation of T cells with a terminally differentiated phenotype has also been described. These cells share phenotypic features with effector memory cells being $CD69^{-}CD25^{-}CD62L^{low}CD127^{low}CCR7^{low}CD27^{low}CD28^{low}$, but they re-express CD45RA (normally restricted to naïve T cells) and thus have been termed T effector memory cells, re-expressing CD45RA (T_{EMRA}) or CD45RA-revertant memory T cells (T_{REV}) (Nikolich-Zugich, 2008). These cells were originally described as exhausted, quiescent cells near the end of their replicative lifespan that could execute immediate effector functions but exhibited severely dysfunctional proliferative responses (Champagne et al., 2001; Geginat et al., 2003). They were thought to allow maintenance of specific immune responses without proliferation that could otherwise drive their terminal exhaustion (Dunne et al., 2005). However, more recent data using a specific peptide stimulus in the presence of exogenous interleukin-2 (IL-2), rather than an anti-CD3 one, suggest that T_{REVS} maintain a high proliferative capacity (van Leeuwen et al., 2002; Wills et al., 2002), which has been supported by *in vivo* observations (Gamadia et al., 2004). Differential costimulation requirements have been shown to be key in explaining these distinct T_{REV} proliferative responses, with T_{REV}s lacking CD28 and the Tumour Necrosis Factor (TNF) Receptor family member CD137L (41-BBL, see section 1.5.1.3) being a critical costimulatory ligand for induction of their proliferation (Waller et al., 2007). Moreover, T_{REV}s have

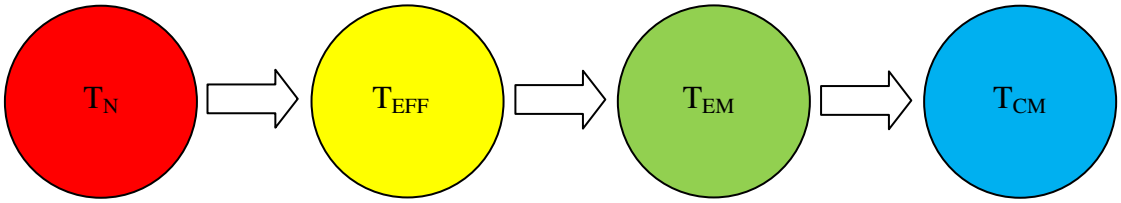
relatively long telomere lengths, at least in young donors (Plunkett et al., 2005). Furthermore, their functional importance is highlighted by the association of both the proportion and absolute numbers of Human Immunodeficiency Virus (HIV) (Northfield et al., 2007) and Cytomegalovirus (CMV) (Lilleri et al., 2008) specific $CD8^+$ T_{REV}s with improved viral control, despite the absence of such a consistent link with the overall magnitude of the HIV specific $CD8^+$ response (Northfield et al., 2007). In summary, these data argue that rather than being a dysfunctional end stage population, T_{REV}s can mediate strong antiviral responses *in vivo*. Nevertheless, this scenario may not be applicable in aged populations whose T_{REV}s possess very short telomeres and may thus be dysfunctional and close to replicative senescence (Akbar and Fletcher, 2005).

1.2.3.1.6 Effector and memory subpopulation differentiation pathways

The differentiation pathways and factors driving lineage decisions between these effector and memory T cell subpopulations remain incompletely understood. Initial models proposed a fixed linear differentiation pathway whereby memory cells arise as direct descendants of effector cells (Figure 1.01A) (Wherry et al., 2003). Alternatively, more recent divergent models predict that memory and effector cells arise as separate lineages (Figure 1.01B) possibly dependent on antigen signal strength (Lanzavecchia and Sallusto, 2002) or as a result of asymmetric division of naïve cells (Chang et al., 2007). Evidence to support both linear and divergent differentiation models currently exist, as reviewed in (Kalia et al., 2010). The re-expression of CD45RA on memory cells appears to be a consequence of homeostatic proliferation in the absence of antigen (Geginat et al., 2003; Dunne et al., 2005; Lilleri et al., 2008; Carrasco et al.,

2006; Sallusto et al., 2004). However, the origins of these CD45RA-revertant memory T cells are also unclear with both central (Geginat et al., 2003; Dunne et al., 2005) and effector memory (Lilleri et al., 2008) populations being proposed as their source.

A. Linear Differentiation Model



B. Divergent Differentiation Model

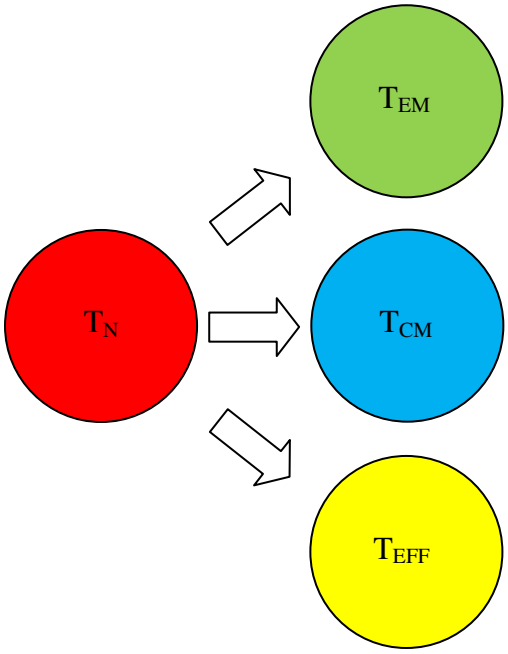
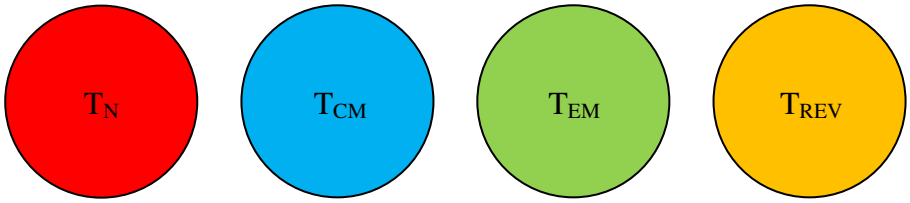


Fig 1.01 Models of Memory T cell Differentiation

1.2.3.1.7 Identification of T cell subpopulations

These distinct T cell subsets are characterised by functional and phenotypic differences that can be defined on the basis of their heterogeneity in antigen expression, as illustrated in Table 1.02. Naïve T cells express the CD45 high molecular weight isoform CD45RA; their differentiation to memory cells coincides with a loss of CD45RA expression, which is replaced by its low molecular weight isoform CD45RO (Sallusto et al., 2004; Schiott et al., 2004). Naïve T cells also express lymph node homing receptors, such as CCR7 and CD62L, which are expressed on central memory cells but lost on the peripherally residing effector memory cells. Although the complexity of CD62L expression limits its usefulness as a phenotypic marker (Sallusto et al., 2004; Lefrancois, 2006; Fierro et al., 2008; Schiott et al., 2004), the loss of CCR7 has been widely used to distinguish central and effector memory populations, so that naïve cells are defined as $CCR7^{+}CD45RA^{+}$, central memory cells as $CCR7^{+}CD45RA^{-}$ and effector memory cells as $CCR7^{-}CD45RA^{-}$ (Lanzavecchia and Sallusto, 2000; Sallusto et al., 1999). Revertant memory T cells re-express CD45RA and lack lymphoid homing markers and therefore can be identified as a $CCR7^{-}CD45RA^{+}$ population. The pattern of CD27 expression is largely superimposable to that of CCR7 (Fierro et al., 2008) however CD27 staining better delineates the positive and negative populations. Thus CD27, alongside CD45RA, has been suggested as the most useful antigen combination to identify the four main T cell subsets: $CD27^{+}CD45RA^{+}$ (naïve, T_N), $CD27^{+}CD45RA^{-}$ (central memory, T_{CM}), $CD27^{-}CD45RA^{-}$ (effector memory, T_{EM}) and $CD27^{-}CD45RA^{+}$ (revertant memory, T_{REV}) (Fierro et al., 2008).



	Naïve	Central Memory	Effector Memory	Revertants
CD27	Hi	Hi	Lo	Lo
CCR7	Hi	Hi	Lo	Lo
CD62L	Hi	Hi	Lo	Lo
CD45RA	Hi	Lo	Lo	Hi
CD45RO	Lo	Hi	Hi	Lo
CD28	Hi	Inter	Lo	Lo
Telomere length	Hi	Inter	Lo	Inter/Lo

Table 1.01 Phenotypic comparison of $CD8^+$ T cell subpopulations

1.2.3.2 Effects of advancing age on the T cell compartment

1.2.3.2.1 Overview

Although peripheral T cell numbers do not diminish with age (Pawelec et al., 2009a) the peripheral T cell pool undergoes a striking age-associated remodelling, with a diminution in naïve T cells and TCR diversity accompanied by an age-associated accumulation of more differentiated memory cells, which accumulate oligoclonal expansions (Pawelec et al., 2010c). Furthermore, these memory T cells display phenotypic changes such as CD28 loss which has been reported to be a key predictor of immune incompetence in the aged (Vallejo, 2005). These age-associated changes are much more strikingly observed amongst the $CD8^+$ T cell compartment than the $CD4^+$s, experiencing clonal expansions and CD28 loss at a far lower frequency (Naylor et al., 2005; Goronzy et al., 2007; Naylor et al., 2005; Effros et al., 1994).

Age-associated defects are not just limited to αβ T cells, as γδ T cells, which recognise antigen in a non-classical manner, also exhibit age related deficits, being reduced in number with attenuated proliferative responses and increased apoptotic sensitivity amongst old donors (Colonna-Romano et al., 2002).

1.2.3.2.2 CD28 Loss

Indeed, although the number or structure of TCRs (signal 1) do not change with age (Fulop, Jr. et al., 1999), diminution of CD28 expression, (signal 2) is one of the most profound and consistent age-associated human immune alterations. At birth virtually all human T cells express CD28 but by the age of 80, 10-15% of peripheral blood $CD4^+$ and over half of $CD8^+$ T cells lack CD28 expression (Weng et al., 2009). Following normal antigenic stimulation, CD28 expression drops but rapidly returns to the same level as before stimulation, but repeated or sustained stimulation can drive CD28 expression loss, along with homeostatic proliferation (Chiu et al., 2006) and inflammation (Bryl et al., 2001). In comparison with $CD28^+$ cells, $CD28^-$ T cells exhibit reduced TCR diversity, shorter telomeres, defective telomerase and proliferative responses and are pro-inflammatory and highly cytotoxic (Weng et al., 2009). Their functional significance is highlighted by their accumulation being associated with impaired immune responses (Goronzy et al., 2001; Saurwein-Teissl et al., 2002). This deficiency is further exacerbated by the impaired induction of its ligands B7.1 and B7.2 amongst the aged (Pawelec et al., 2010c), so that even cells that still express CD28 may exhibit diminished responses. However, the idea that CD28 loss is a critical factor in the dysfunction of $CD28^-CD8^+$ T cells has been challenged by the finding that restoration of CD28 signalling could not reverse their

low telomerase activity (whose significance is explored below) (Plunkett et al., 2007), though this finding itself has recently been disputed (Effros, 2010).

1.2.3.2.3 Cellular senescence

Naïve T cell loss and CD28 diminution has only been demonstrated to partially explain the inability to adequately activate T cells from old donors, which also reflects age-associated deleterious alterations on a per cell basis (Maue et al., 2009; Eaton et al., 2004; Pfister et al., 2006). Indeed, this may be a consequence of cellular senescence, defined as the irreversible loss of the replicative potential of somatic cells, which can be induced in a telomere dependent or telomere independent manner

1.2.3.2.3.1 Telomeres

The complete replication of linear chromosomes is problematic for the eukaryotic DNA replication machinery as it requires a 5' primer for the initiation of DNA synthesis. This 'end replication problem' (Osterhage and Friedman, 2009) prevents complete replication of the 3' end of chromosomes, producing a single DNA strand that is unstable, resulting in the loss of 50-200 base pairs of DNA with each cell division (Kaszubowska, 2008). To overcome this problem, linear chromosome ends are composed of non-coding double stranded repeat sequences, known as telomeric DNA, that protect DNA ends from erosion. This DNA is associated with a protein complex to form capped structures known as telomeres (Andrews et al., 2009). These prevent chromosome ends undergoing illegitimate recombination, fusion events and

the possibility of being recognised by cellular repair systems as double stranded DNA breaks.

Telomeres erode with each somatic cell division and cells can only undergo a finite number of cell divisions (the Hayflick number (Hayflick and Moorhead, 1961)) before their telomeres reach a critically short length and uncap (Verdun and Karlseder, 2007). This results in a DNA damage response (DDR) signal being transmitted that temporarily arrests cell growth whilst the lesion is repaired (Campisi and d'Adda di, 2007; d'Adda di et al., 2003). If the damage is irreparable, prolonged DDR signalling ensues that triggers either apoptosis or an irreversible state of growth arrest termed replicative senescence, dependent upon the cell type (Blackburn, 2005; Verdun and Karlseder, 2007). Independently of telomere erosion, damage anywhere in the genome, oncogene expression and cellular stress can all induce a DDR that results in senescence (Campisi and d'Adda di, 2007). The presence of critically short sentinel telomeres rather than the mean cellular telomere length is critically important to initiate this process (Hemann et al., 2001). *In vivo*, cellular senescence prevents incipient cancer cells proliferating, thereby forming a critical means of inhibiting tumourigenesis (Campisi, 2005). However, amongst aged tissues, the accumulation of senescent cells can perturb normal tissue homeostasis, compromise regenerative capacity and promote ageing (Tyner et al., 2002; Hornsby, 2002; Kim and Sharpless, 2006).

1.2.3.2.3.2 Telomerase

Germ cell telomeres, in contrast to somatic cells, do not undergo attrition with every cell division because they are maintained by the reverse transcriptase activity of the enzyme telomerase (Hiyama et al., 1995). However, lymphocytes are unique amongst somatic cells in that they can upregulate telomerase during entry into the cell cycle that can initially compensate for replication associated telomere loss (Hiyama et al., 1995; Igarashi and Sakaguchi, 1997). Nevertheless, the ability of T cells to upregulate telomerase progressively diminishes following repeated activation (Valenzuela and Effros, 2002; Roth et al., 2003)

Telomerase induction in lymphocytes is primarily regulated through PI3K/Akt, which directly phosphorylates and activates hTERT, the catalytic unit of telomerase (Akiyama et al., 2004). Furthermore, Akt, alongside PKC, activates NF-κB, which mediates the nuclear translocation and indirect activation of hTERT (Sheng et al., 2006; Akiyama et al., 2004). PI3K/Akt pathway is a key downstream mediator of CD28 costimulatory signals (further discussed in section 1.5.1.1) and following repetitive stimulation, T cells lose CD28 expression (as described in section 1.2.3.2.2), which mirrors their loss of telomerase induction. However, restoration of CD28 signalling in $CD28^{-}CD27^{-}CD8^{+}$ T cells could not reverse their telomerase impairment (Plunkett et al., 2007). This, instead, was associated with defective phosphorylation of the key signalling kinase Akt at Ser^{473} (Plunkett et al., 2007), which is one of two phosphorylation sites that control its activation (Jacinto et al., 2006; Alessi et al., 1996). Nevertheless, blocking E-cadherin significantly reversed their defective Akt phosphorylation, but these cells remained refractory to telomerase induction (Henson

et al., 2009), indicating that other, Akt independent, mechanisms are involved in their telomerase downregulation.

1.2.3.2.3.3 Significance of cellular senescence in immune system ageing

The adaptive immune system is particularly sensitive to the effects of replication associated telomere loss, as its competence is critically dependent upon lymphocytes undergoing massive clonal expansion upon encountering cognate antigen (Akbar et al., 2004; Kaszubowska, 2008; Andrews et al., 2009). Their age related loss of telomerase induction results in lymphocytes exhibiting progressive telomere attrition (Plunkett et al., 2001; Hodes et al., 2002; Rufer et al., 1999; Son et al., 2000), whose significance is highlighted by their shortened telomeres being predictive of earlier mortality (Cawthon et al., 2003) and associated with a variety of age-associated pathologies (Calado and Young, 2009).

As well as irreversible growth arrest, most senescent cells acquire apoptotic resistance (Campisi and d'Adda di, 2007), which may account for their age-associated accumulation (Passos et al., 2010). By contrast, lymphocytes from old, as compared with young, humans exhibit an increased propensity to undergo apoptosis following a wide variety of mitogenic stimuli (Herndon et al., 1997; Potestio et al., 1998). Nevertheless, this may largely reflect the apoptotic process involved in deletion of effector cells following infection resolution, by a process termed activation induced cell death (AICD). AICD is mediated through Fas/FasL interactions and, indeed, lymphocytes from aged humans are more sensitive to apoptosis induced by the Fas

and TNFα death receptor pathways than their young counterparts (Aggarwal and Gupta, 1998; Aggarwal et al., 1999; Salvioli et al., 2003). However, these extrinsic apoptotic mechanisms likely differ from the intrinsic pathway, which is induced following a wide variety of stimuli that include oxidative stress and DNA damage (Monti et al., 2000). Indeed, human EBV specific $CD8^+$ T cells repeatedly stimulated to divide in culture develop growth arrest and become resistant to apoptosis in response to a variety of stimuli that strongly induced apoptosis in their less differentiated counterparts (Spaulding et al., 1999). Moreover, human T_{EM} and T_{REV} $CD8^+$ T cells, whose numbers increase with age (as described earlier), are more resistant to oxidative stress, calcium and TNFα induced apoptosis than their T_N and T_{CM} counterparts (Gupta and Gollapudi, 2006; Gupta, 2005) and lymphocytes from human donors of advanced age are more resistant to apoptosis under conditions of oxidative stress compared with the young (Monti et al., 2000). Furthermore, the age related accumulation of oligoclonal CMV specific $CD8^+$ T cell expansions (detailed in section 1.4.3.3.2) results from their extended lifespan (Wallace et al., 2010), suggesting such cells become senescent, acquiring apoptosis resistance. Nevertheless, unlike fibroblasts and epithelial cells, following a DDR lymphocytes preferentially undergo apoptosis (Campisi and d'Adda di, 2007). However, lymphocytes can still become senescent (Schmitt et al., 2004; Vallejo et al., 2004; Montes et al., 2008; Aoshiba and Nagai, 2009; Simpson et al., 2010; Plunkett et al., 2005) and even if this only reflects a small proportion of lymphocytes undergoing a DDR, their rapid turnover (Hellerstein et al., 1999) would manifest itself as an age related accumulation. Senescent lymphocytes also remain metabolically active, exhibiting widespread changes to gene expression unrelated to proliferation, most notably up-regulating pro-

inflammatory secretory genes (Fumagalli and d'Adda di, 2009), which may underlie the development of the age related subclinical pro-inflammatory state termed 'inflammaging.'

1.2.3.2.4 Inflammaging

Multiple studies have also demonstrated that the age relate decline in immune cell activity is at least in part mediated by the accumulation of factors in the serum of old animals, likely including cytokines alongside, for example, hormones (Gomez et al., 2006; Bagnara et al., 2000; De et al., 2004). In particular, the *in vitro* addition of exogenous IL-2 can rejuvenate many lymphocyte functions of the aged including proliferation, differentiation and cytotoxicity defects (Haynes et al., 2000; Haynes and Eaton, 2005).

Indeed, the complex immune system remodelling observed during ageing includes a well characterised profound modification of the cytokine network. A key feature of this phenomenon is an increase in pro-inflammatory molecules alongside a decrease in tropic agents (e.g. IL-2). Murine studies generally support an age related shift from a Th1 like (IL-2, IFNγ) to a predominantly Th2 like (IL-4, IL-6, IL-10) cytokine response (Shearer, 1997; Segal et al., 1997). However, a meta-analysis of over 60 human studies revealed no such consistent change (Gardner and Murasko, 2002).

Ageing is associated with an increase in plasma levels and the capability of leukocytes to produce inflammatory components including TNFα (Bruunsgaard et al., 1999; Paolisso et al., 1998), IL-6 (Cohen et al., 1997) and acute phase proteins such as

CRP (Ballou et al., 1996). These circulating inflammatory parameters can be positively correlated, suggesting a generalised activation of the entire inflammatory network (Bruunsgaard et al., 1999). However, the observed 2-4 fold increase in circulating inflammatory parameters is far from levels attained in acute infection (Bruunsgaard et al., 2001). Therefore, ageing is said to be associated with a low grade chronic pro-inflammatory condition termed 'inflammaging' (Franceschi et al., 2000b).

Inflammaging may be driven by the chronic exposure of macrophages, the central source of these pro-inflammator molecules, to a variety of stressors such as reactive oxygen species (ROS) from endogenous metabolic pathways or antigens, leading to their systemic activation (Franceschi, 2007; Franceschi et al., 2000b). Alternatively, the age-related increase in other pro-inflammatory cells such as T cells lacking CD28 expression may underpin inflammaging (Weiskopf et al., 2009). Indeed, these cells are augmented amongst CMV seropositive donors and, as the dominant chronic stressor, CMV antigens may mediate systematic macrophage activation and exacerbate inflammaging. Indeed, CMV seropositivity is associated with an upregulation of a variety of pro-inflammatory agents (Mogensen and Paludan, 2001; Varani et al., 2009; Tong et al., 2001). Additionally, ageing is correlated with increased adiposity and adipiocytes can secrete large quantities of pro-inflammatory adipokines and cytokines (Tchkonia et al., 2010), which recruit and activate macrophages to a classically activated phenotype that further propagates the inflammatory response (Maury and Brichard, 2010).

Although inflammation is a process critical for dealing with infections and tissue damage, inflammaging appears to be physiologically deleterious and is predictive of all-cause mortality in multiple elderly cohorts (Krabbe et al., 2004). Moreover, inflammation is a characteristic part of the pathological processes of many of the most prevalent and severe age related pathologies, including cardiovascular diseases, rheumatoid arthritis and Alzheimer's, whose development are further associated with inflammaging (reviewed in Macaulay *et al*, manuscript submitted).

1.2.3.2.5 Other age-associated T cell alterations

Aged humans also exhibit a compromised ability to effectively form immunological synapses (Tomoiu et al., 2007). These result from a dynamic lipid raft reorganisation at the TC-APC interaction site that segregates positive and negative activators of T cell activation, generating an environment for the amplification of signalling transduction cascades. Impairment in their generation diminishes the signalling intensity, and thus impairs the ability of T cells to activate, expand and differentiate (Garcia and Miller, 2002). A major factor underlying this defect is the increased cholesterol content of cell membranes of the aged, increasing membrane rigidity and impairing membrane raft functionality (Tomoiu et al., 2007), which may in part explain the multiple deleterious alterations in TCR signalling cascades observed in aged T cells. Indeed, increasing the membrane cholesterol content of young T cells to that of old donors rendered them functionally aged and conversely decreasing cholesterol of aged T cells improved their IL-2 production and proliferation (Tomoiu et al., 2007).

1.3 What causes these age related T cell changes?

These T cell changes are considered biomarkers of human immune system ageing and are precipitated by two key events: 1) decline in lymphocyte generation resulting from thymic involution and 2) continuous attrition caused by lifelong chronic antigen burden.

1.3.1 Thymic Involution

1.3.1.1 Introduction

The thymus plays a crucial role in T cell development, providing a specialised microenvironment in which hematopoietic bone marrow derived precursors are able to replicate, mature and undergo strict selection procedures to shape a T cell repertoire that is sufficiently diverse and sensitive towards the vast array of foreign antigens but is also non-reactive to self (Sebzda et al., 1999).

The gene rearrangement process that generates TCRs is random. It may thus result in ineffective T cells which bind too weakly to MHC to recognise antigenic peptide or autoreactive T cells that bind strongly to self-MHC peptide complexes and such cells are eliminated in the thymus by the processes of positive and negative selection, respectively (Klein et al., 2009). Only interactions with intermediate affinity allow lineage commitment and export into the periphery, resulting in the deletion of the vast majority (~97%) of thymocytes (Klein et al., 2009). Surviving cells are exported into the periphery as recent thymic emigrants, which mature post thymically to form naïve T cells (Houston, Jr. et al., 2008).

1.3.1.2 Age-associated thymic changes

The human thymus regresses (or involutes) progressively throughout life with its functional tissue becoming gradually replaced with adipocytes, to the extent that this organ becomes barely identifiable in adults. This constitutes one of the most ubiquitous, dramatic and recognisable changes during ageing (Steinmann et al., 1985).

1.3.1.3 Homeostatic peripheral expansion

Nevertheless, considering 1% of the total T cell pool has been estimated to be replenished each day, equating to 3×10^9 T cells (Goronzy et al., 2007) and thymopoiesis is the exclusive mechanism by which novel T cells may be generated, the total size of the T cell pool changes remarkably little with advancing age (Pawelec et al., 2009a). However, T cells can also be replenished extra-thymically, by a process termed homeostatic proliferation, that is independent of antigen, which already significantly contributes towards building the T cell repertoire in neonates, with over half of all foetal T cells being generated homeostatically (Schonland et al., 2003). Moreover, thymectomised infants appear to build a normal T cell compartment with no reported increased frequencies of major infections (Goronzy et al., 2007). However, such individuals also exhibit signs of a prematurely aged immune system (Sauce et al., 2009), suggesting that although homeostatic proliferation is the major mechanism of T cell production in adults, thymic export still makes a significant contribution. Nevertheless, homeostatic proliferation may itself have physiological limitations. Indeed, this peripheral expansion depends on self-recognition and may be subject to selection pressures that compromise TCR diversity over time (Goronzy and Weyand,

2005). Furthermore, homeostatic proliferation can induce the differentiation of naïve to memory T cells (Ge et al., 2002), which would drive attrition of the naïve T cell pool and loss of TCR repertoire diversity.

1.3.1.4 TCR Repertoire Diversity

The receptor diversity of the naïve T cell compartment is critical to the ability to mount a T cell response to novel antigenic challenges such as those provided by newly emerging viruses, such as SARS, and by antigenic drift of existing viruses, such as influenza, both of which disproportionately affected the elderly in terms of incidence and severity (Naylor et al., 2005).

The enormous TCR repertoire diversity reflects the highly polymorphic nature of the TCR. The TCR is a heterodimeric protein that consists of two polypeptide chains (α and β) each containing one variable, V and one constant, C domain. The CDR3 (third complementary-determining region) loops of the α and β chain V domains is largely responsible for the peptide specificity of the TCR (Robins et al., 2009). These CDR3 loops are formed by recombination between multiple V, D and J gene segments in the β chain locus and between analogous V and J segments in the α locus, which allow a large number of distinct CDR3 sequences to be encoded and the diversity is further increased by addition and deletion of nucleotides at the recombination junctions during TCR gene rearrangement (Robins et al., 2009).

A theoretical estimate of TCR diversity generated by this system is of the order of 10^{15} but intrathymic selection processes limit the generated repertoire to around 10^{13}

(Nikolich-Zugich et al., 2004). However, the human T cell system is estimated to consist of approximately $3x10^{11}$ cells with a naïve T cell diversity of 10^7-10^8 different TCRs, with each T cell specificity being represented by a clone of at least 1000 cells (Robins et al., 2009). By comparison, the T cell repertoire of the memory pool is contracted by an estimated factor of 100 (Nikolich-Zugich et al., 2004).

Maintenance of the TCR repertoire is seen as critical to ensure responses to a wide range of antigenic challenges. Indeed, a twofold reduction in murine TCR diversity is sufficient to generate holes in the repertoire to various antigens (Woodland et al., 1990) and the human TCR repertoire can be closely correlated with remaining lifespan amongst the very old (Hadrup et al., 2006). Moreover, through interclonal competition, clonal diversity limits inappropriate T cell expansions and its loss may promote autoimmunity (Goronzy and Weyand, 2003).

1.3.2 Lifelong antigenic burden

It has been shown that immune alterations typically associated with advanced age are observed amongst young individuals who have been subjected to a high antigenic burden. This is demonstrated in healthy African populations who are known to live in an environment in which the immune system is continuously challenged (van Baarle et al., 2005; Miles et al., 2007; Ben-Smith et al., 2008), as well as HIV infected individuals (Naeger et al., 2010; Desai and Landay, 2010; Appay et al., 2007; van Baarle et al., 2005), patients with X-linked proliferative syndrome (XLP) (Plunkett et al., 2005) (who undergo uncontrolled lymphocyte expansion in response to primary

Epstein-Barr virus (EBV) infection (Nelson and Terhorst, 2000)) and subjects affected by the long term persistent antigen stimulation provided by cancer (Pawelec et al., 2006) or autoimmune diseases (Thewissen et al., 2005; Thewissen et al., 2007). These data suggest that lifelong antigen burden rather than ageing *per se* may be the primary factor driving extreme differentiation of the T cell pool.

1.3.2.1 Persistent poorly controlled antigenic challenges

Very large antigenic loads are incurred by the host immune system in response to persistent viral infections, such as HIV and Hepatitis C virus (HCV). In such infections, the virus continually replicates in the face of an ongoing immune response, continuously and systematically stimulating and exhausting the virus specific T cells, such that they typically cause pathology resulting in chronic diseases with poor prognoses. Therefore, although such diseases incur a high antigenic load in the host, they are far from ubiquitous, affecting only a small minority of the old and causing premature mortality in the affected young. Thus they are not considered to be contributory towards the typical ageing process and such infections will not be further discussed, except to draw parallels between the functional consequences of such infections and those that result from T cell ageing.

1.3.2.2 Latent infections

More likely candidates that could contribute towards age-associated immune decline are latent infections. In contrast to persistent viral infections, these are systemically controlled after initial infection but instead of being cleared from the host, as in acute viral infections, the virus finds a niche where it can establish a quiescent latency.

Latency is a unique transcriptional and translational viral state whereby the productive replicative cycle and hence the expression of most or all antigens is silent but their genome persists. Such viruses must intermittently enter the lytic part of their life cycle and reactivate to spread both within and between hosts. Thus, by virtue of their repeated lifelong reactivation without causing overt disease, latent viruses may repetitively stimulate the immune system and play a role in driving age-associated immune changes

The best studied latent viruses in this context are the herpesviridae. Of these, CMV in particular appears to act as the dominant chronic stressor, being highly ubiquitous and associated with many of the same phenotypic and functional alterations to T cell immunity, collectively known as the Immune Risk Phenotype (IRP), that have been suggested as biomarkers of immune system ageing (Wikby et al., 2002; Olsson et al., 2000) (further discussed in section 1.4.2). The significance of CMV infection in health outcomes of elderly individuals is further highlighted by its association with deleterious responses to influenza vaccination (Trzonkowski et al., 2003), heterologous infection (Griffiths, 2006; Khan et al., 2004) and cancer (Pawelec et al., 2010b). Moreover, the immune response towards CMV is very large in magnitude and progressively expands with age such that the T cell pool of old donors is frequently dominated by large, usually CMV specific, dysfunctional T cell clones, (further discussed in section 1.4.3.3.2). This 'memory inflation' is also a feature of persistent HIV and HCV infections but not EBV or Varicella Zoster Virus (VZV) (Brunner et al., 2010).

1.3.2.3 Other chronic pathogens

However, not everyone is CMV positive even at advanced age but still exhibit characteristics of immunosenescence. Indeed, each human individual harbours an estimated 8-12 chronic infections (Virgin et al., 2009), which may also play roles in driving age-associated immune decline. A good candidate amongst CMV negative old individuals is EBV, which infects 99.9% of people and whose T cell responses expand with age only in the absence of CMV infection (Khan et al., 2004). Additional contributions may be made by the polyomavirus family, which consist of five viruses that share many features with herpesviruses that are pertinent to T cell ageing, including benign primary infection at an early age, high prevalence that only causes pathological consequences when there is concomitant immuno-suppression and reactivation with increasing frequency amongst the aged (Zhong et al., 2007; Kean et al., 2009; Nguyen et al., 2009; Sharp et al., 2009). Furthermore, in other environments, intestinal parasites, which can produce millions of eggs and copious excretory/secretory products that persistently stimulate the host immune system (Kassu et al., 2003), may play a similar role in driving age-associated immuno-dysfunction. Indeed, infected subjects displayed substantial alterations to immune parameters similar to those described as CMV or age-associated, which were largely reversible upon parasite treatment (Kalinkovich et al., 1998). Other candidates include anelloviruses, the adeno-associated virus (AAV) (Virgin et al., 2009) and human herpes viruses 6 (HHV-6) and HHV-7 (Moss, 2010). Nevertheless, many of the aforementioned pathogens' impact on the ageing process remain poorly explored and CMV remains the only pathogen to be associated with the immune risk profile.

1.4 CMV

1.4.1 Introduction

1.4.1.1 Background

CMV is a member of the β-subfamily of the herpesviridae, which are ancient organisms that have evolved alongside eukaryotic cells for millions of years and possess the ability to remain latent in their hosts after initial infection. Indeed, CMV is frequently cited as one of the most successful human pathogens, being highly ubiquitous (McDonald et al., 2004) with its primary infection widely considered asymptomatic and persisting causing minimal impairment in immuno-competent hosts, enabling them to remain active and maximise the opportunity of encountering other successful contacts. Nevertheless, amongst immnocompromised patients, CMV is one of the most common and severe opportunistic pathogens (Freeman, Jr., 2009; Steininger et al., 2006) and primary infection during pregnancy carries a significant risk of developmental defects and mental retardation in newborns (Tsutsui, 2009).

1.4.1.2 CMV infection routes and dissemination

CMV is able to infect by a number of routes including trans-placentally, intra-uterinally, peri-natally, and post-natally via infected sweat saliva, semen, urine and breast milk; blood transfusion and organ transplantation (Drago et al., 2000). Once *in vivo*, CMV can infect an exceptionally wide range of host cell types, including multiple haematopoietic cell types and the parenchymal and connective tissue cells of virtually any organ (Sinzger et al., 2008). In particular, monocytes and endothelial cells, which recruit and transfer virus to the migrating monocytes, play key roles in

the body wide dissemination of CMV during acute infection (Bentz et al., 2006). CMV pathogenesis results from the direct cytopathic effects of CMV on infected host organs (Bissinger et al., 2002). Additionally, the host immune response may also contribute to tissue damage, through $CD8^+$ T cell mediated lysis of infected cells and the inflammatory nature of effector responses (Moss, 2010).

1.4.1.3 Latency

Monocytes are nevertheless not productive for viral replication, being abortively infected instead. However, since the genome is maintained they are considered to be reservoirs of latent CMV and vehicles for viral dissemination. In contrast, permissive viral infection occurs in macrophages, their differentiated counterparts (Smith et al., 2004). Indeed, CMV infection of monocytes drives cellular activation, motility, endothelial migration and macrophage differentiation (Smith et al., 2004).

1.4.1.4 Reactivation

Monocytes are stimulated to differentiate to permissive macrophages by several cytokines, including the central inflammatory mediators TNFα and Interferon gamma (IFNγ), which also activate the CMV immediate early (IE) promoter region, thereby facilitating active CMV replication (Freeman, Jr., 2009). This enables spreading to other hosts by allowing virus to be shed in secretions associated with the inflammatory response. Moreover, numerous epidemiological studies associate CMV infection with multiple chronic inflammatory disorders, being specifically detected at sites of chronic inflammation, that is thought to reflect inflammation inducing CMV

reactivation, which further exacerbates the inflammatory response (Freeman, Jr., 2009).

1.4.1.5 Mechanisms of Immune Evasion

CMV has the largest DNA genome of any human virus, around 240 kilobase (kB) pairs long, and is capable of encoding more than 200 proteins, but approximately three quarters of these are dispensable for viral replicative functions (Dunn et al., 2003). Indeed, many CMV proteins possess immuno-modulatory functions, producing both pro-inflammatory and wide ranging immuno-suppressive effects (Freeman, Jr., 2009). Examples of immune evasion include directly infecting the initiators of adaptive immunity, the DCs, inducing a so-called 'paralysed' phenotype, characterised by downregulation of MHC and costimulatory molecules and altering their cytokine profile so that they retain the ability to prime naïve T cells but tolerise or delete them rather than activate them (Rolle and Olweus, 2009). However, despite the presence of multi-layered viral evasion mechanisms, an immune response of considerable magnitude is generated against CMV and infection is well controlled in humans, even at an advanced age (Weiskopf et al., 2009). Nevertheless, cross presentation of viral antigens by uninfected DCs can bypass viral escape mechanisms and ensure efficient T cell priming (Benedict et al., 2008). Additionally, most of the studies characterising CMV 'paralysing' DCs (Rolle and Olweus, 2009) were performed on monocyte derived DCs, whereas $CD11c^+$ myeloid DCs do not exhibit a paralysed phenotype and plasmacytoid DCs are resistant to CMV infection (Kvale et al., 2006). These constitute the two major populations of peripheral blood DC subsets and may thus represent the key initiators of anti-CMV immunity.

1.4.2 The Immune Risk Phenotype (IRP)

A longitudinal study of ageing amongst healthy Swedish octogenarians (OCTO immune study) (Olsson et al., 2000) and further refined using a nonagenarian population not selected for good health (NONA study) (Wikby et al., 2002), revealed a cluster of immune parameters associated with poor immune function and predictive of earlier mortality, termed the IRP. The IRP is characterised by an inverted CD4:CD8 ratio, poor T cell mitogen responses, increased levels of $CD28^{-}CD8^{+}$ T cells and low B cell counts. Moreover, CMV infection is associated with, and is suggested to be a major contributor towards, the IRP (Pawelec et al., 2004; Derhovanessian et al., 2009).

1.4.2.1 CMV is associated with the IRP

Although all individuals with an IRP were CMV^{+}, only a small fraction of CMV^{+} individuals possessed an IRP, and no simple direct correlation between CMV and mortality has been observed (Pawelec et al., 2010c). The association of CMV with the IRP is not thought to be linked to the presence of CMV infection alone but reflects the manner in which an individual's immune system deals with the virus (Strandberg et al., 2009; Derhovanessian et al., 2010; Wang et al., 2010). This could be influenced by the duration of infection, whereby a certain time period of infection is required for transition from a non-IRP into the IRP category (Pawelec et al., 2005), the frequency of reactivation, which may increase with age (Stowe et al., 2007) and/or the host inflammatory genetic profile (Derhovanessian et al., 2010).

1.4.2.2 IRP applicability

Whether the IRP can be identified and be predictive of mortality among younger individuals is a crucial question pertaining to its relevance. Indeed, individuals under 40 years of age, have been identified in the IRP but associations with mortality only become apparent above the age of 60 (Wikby et al., 2008), though this may reflect the relative scarcity of mortality and IRP amongst younger cohorts. Moreover, the concept of the IRP emerged from studying relatively small numbers of people who live in a single Swedish municipality, unusual for having a high prevalence of long lived individuals. However, data has emerged from the UK (Huppert et al., 2003) and Brazil (Peres et al., 2003) showing that an inverted CD4:CD8 ratio, the best marker for the IRP, is correlated with reduced survival rates. Nevertheless, the IRP has been applied to younger populations undergoing autoimmune disorders, cancer and HIV, when it is unclear whether it is predictive of mortality under these different circumstances (Pawelec et al., 2010c).

1.4.3 T cell immune response against latent and persistent pathogens

1.4.3.1 Comparing antiviral responses against cleared and persistent antigens

There is a large body of evidence that suggests that the success of antiviral immunity is critically determined by the effectiveness of $CD8^+$ T cell response, particularly in the long term control of persisting and latent viruses (Bangham, 2009). During the acute phase of viral infection the effector T cells generated in response to latent or persistent viruses appear largely similar to those produced in response to acute viral challenge, being activated, proliferating, cytotoxic, peripherally migrating, apoptotic

prone and expressing the phenotypic markers CD27, CD28 and CD45RO (van Lier et al., 2003; Virgin et al., 2009; Appay et al., 2002). However, during an acute virus specific response, the pathogen is typically cleared within 1-2 weeks, after which the majority (around 90%) of these expanded effector cells die by apoptosis, generating functional memory cells persisting independently of antigen and exhibiting slow homeostatic proliferation (as described in section 1.3.1.3). In contrast, during a chronic viral infection, specific $CD8^+$ memory T cells arise which are completely dependent on antigen (alongside IL-2) for their maintenance, undergo extensive antigen driven proliferation and display multiple dysfunctions, including defective cytokine production, cytolysis and proliferative responses (Wiesel et al., 2009). These differences between acute and chronic virus specific cells are thought to be a consequence of antigen persistence versus antigen clearance (Sabbaj et al., 2007; Shin and Wherry, 2007; Mueller and Ahmed, 2009; Bucks et al., 2009). Indeed, exhausted $CD8^+$ T cell responses have also been observed in response to the persistent antigenic stimulation provided by tumours (Kim and Ahmed, 2010) and parasite infections (Joshi et al., 2009; Hernandez-Ruiz et al., 2010). It should also be noted that exhaustion has also been observed amongst $CD4^+$ T cell (Brooks et al., 2005) and B cell responses (Moir et al., 2008). Nevertheless, exhaustion and senescence (as described in section 1.2.3.2.3) are separate physiological phenomena and a table comparing their similarities and differences is depicted below: -

	Senescence	**Exhaustion**
Definition	Growth arrest resulting from cellular turnover or stress	Persistence of virus specific T cells lacking effector functions
Induction	Progressive telomere shortening with	Antigen persistence following acute

	each cell division (telomere dependent) DNA damage/cellular stress (telomere independent)	infection giving rise to 'antigen addicted' T cells
Cellular Effects	Proliferative inability Apoptosis resistance Loss CD28 Decreased IFNγ Increased pro-inflammatory cytokines	Exhaustion comprises spectrum of dysfunction whereby functional responses are lost in a hierarchical manner with Cytotoxicity and IL-2 compromised early TNFα lost at an intermediate stage and IFNγ being the most resistant Proliferative impairment being increasingly pronounced across the exhaustion gradient
Physiological Function	Inhibit tumourigenesis	Prevent auto-immunity
Signalling	p38MAPK→p53→p21	PD-1→ inhibits PI3K/Akt
Irreversible?	No, p38 inhibition can restore function	No, blocking PD-1/PD-L interactions restores function

Table 1.02 Comparison of T cell exhaustion and senescence

Senescence results from a DNA damage response (DDR) that activates the checkpoint proteins p53 and p21 through the MAPK signalling molecule p38 (Passos et al., 2010; Lansdorp, 2000). Blocking these key signalling mediators can reverse senescence at an early stage but prolonged DDR signalling ultimately leads to irreversible senescence (Passos et al., 2010; d'Adda di, 2008; Rodier et al., 2009; Shiloh, 2003; Beausejour et al., 2003; Lansdorp, 2000; Davis et al., 2007; Brown et al., 1997). Exhaustion is also reversible with the interruption of PD-1/L interactions augmenting functions (further discussed in section 1.5.3.2.3), though highly exhausted cells co-express additional inhibitory receptors and may be refractory to PD-1/L blockade alone (Blackburn et al., 2008; Nakamoto et al., 2008).

1.4.3.2 Exhaustion gradient

Although functional exhaustion of virus specific $CD8^+$ T cells has been demonstrated in a wide range of human persistent and latent antigenic challenges, considerable heterogeneity exists in the degree of dysfunction observed in these reports not only between different diseases but also between individuals with the same virus and also amongst cells specific for the same virus within a single donor (Wherry and Ahmed, 2004). Indeed, exhaustion is not an all or nothing phenomena but instead comprises a spectrum of dysfunction whereby functional responses are lost in a hierarchical

manner with cytotoxicity and IL-2 production compromised early, TNFα lost at an intermediate stage and IFNγ being the most resistant to exhaustion (Shin and Wherry, 2007). Proliferative impairment is a key feature of exhaustion, occurring when other functions are intact, and becomes increasingly impaired across the exhaustion gradient (Freeman et al., 2006). Fully exhausted cells demonstrate complete loss of all effector functions and in the extreme situation, exhausted T cells can undergo apoptotic deletion (Freeman et al., 2006; Virgin et al., 2009). Furthermore, the functional importance of this exhaustion hierarchy is reflected in that the level of exhaustion, rather than the size of the response, can be correlated with inability of the immune system to control the virus (Bangham, 2009). The force driving this loss of function is antigen load (Shin and Wherry, 2007; Mueller and Ahmed, 2009). However, this relationship is a complex one and also depends upon the (1) latent or persistent nature of the virus, (2) viral tropism, (3) epitopes presented and (4) presence of $CD4^+$ T cell help (Wherry and Ahmed, 2004).

$CD8^+$ T cells can cause massive host tissue damage by virtue of their cytotoxicity and can proliferate at an extraordinary rate, thus functional inactivation in the presence of persisting antigen may represent a mechanism of peripheral tolerance (Virgin et al., 2009). Indeed, in the hierarchy of T cell exhaustion these potentially harmful responses are silenced first. Most pathogenic challenges are cleared after primary infection, thus persisting antigen may normally be a sign of an autoimmune response. However, many evolutionarily ancient chronic viruses co-exist with the host without being significantly detrimental and thus immune responses may result in immunopathological damage for very little benefit (Barber et al., 2006).

1.4.3.3 CMV specific immune response

1.4.3.3.1 Comparing CMV with other chronic viruses

Although highly similar during primary infection, over subsequent months during the chronic phase of infection, $CD8^+$ T cells specific for a particular virus tend to develop a predominant phenotype and level of dysfunction. Indeed, HCV and influenza specific $CD8^+$ T cells tend to display an early, HIV and EBV exhibit an intermediate and CMV mainly a late differentiated phenotype (Appay et al., 2002). Moreover, HIV and HCV specific $CD8^+$ T cells are profoundly dysfunctional whereas EBV specific cells retain a high degree of function (Virgin et al., 2009); CMV specific $CD8^+$ T cells lie somewhere in between these two ends of this spectrum. Indeed, although CMV specific $CD8^+$ T cells clearly exhibit protective capacities (Holtappels et al., 2008), they display highly impaired IL-2 (Almanzar et al., 2004) and IL-7R expression (Sauce et al., 2007b) and an age-associated impairment in IFNγ production has also been reported (Ouyang et al., 2003a). This suggests CMV specific $CD8^+$ T cells exhibit a moderate degree of functional exhaustion which worsens with age.

1.4.3.3.2 Memory inflation

In direct contrast to the T cell response observed towards most other pathogens, the magnitude of the CMV immune response undergoes progressive long term expansion with age (Nikolich-Zugich, 2008; Fletcher et al., 2005) that has been estimated to occupy between 10% and 40% of the $CD4^+$ T cell pool (Pourgheysari et al., 2007; Sylwester et al., 2005; Sester et al., 2002). Moreover, $CD8^+$ T cell responses, directed

against a sole epitope of a single CMV protein, can occupy over a quarter (Khan et al., 2002; Ouyang et al., 2003b) and up to 40% (Khan et al., 2004) of the entire $CD8^+$ compartment of the elderly. Their progressive expansion is associated with an accumulation of increasingly dysfunctional and differentiated CMV specific $CD8^+$ T cell clones (Ouyang et al., 2003a), which may out-compete other T cell populations for immunological space, resulting in loss of immunological memory to previously controlled pathogens and further constrictions in the TCR repertoire (Akbar and Fletcher, 2005; Khan et al., 2004; Trzonkowski et al., 2003). Indeed, their significance is highlighted by their association with reduced lifespan amongst aged individuals (Hadrup et al., 2006). Furthermore, humoural anti-CMV responses also significantly intensify with advancing age and their magnitude is correlated with functional and cognitive impairments and co-morbidity (Vescovini et al., 2010). Thus, the increasing weight of immune resources dedicated to the control of CMV may significantly impair immune responses amongst the aged.

1.4.3.3.3 T cell clonal diversity

An important feature of the immune system is the ability to generate a multi-pronged T cell response that can recognise a diverse repertoire of antigens presented by MHC molecules (epitope diversity) and also the diversity of TCRs amongst T cells that recognise the same peptide-MHC complexes (clonotypic diversity) (Pewe et al., 2004). Indeed, their functional significance is highlighted by the breadth of a $CD8^+$ T cell response being correlated with the success of chronic viral control (Lauer et al., 2004; Day et al., 2002; Sacre et al., 2005; Messaoudi et al., 2004). A diverse T cell response both in terms of clonality and epitope may enable the host to select the highest avidity

TCR and thus produce an optimal response (Nikolich-Zugich et al., 2004). Additionally, it may prevent escape mutants arising in immunodominant viral epitopes (Price et al., 2004; Barouch et al., 2005) and avoid holes in the T cell repertoire that arise as a consequence of ageing or clonal exhaustion (Nikolich-Zugich et al., 2004).

The cellular immune response directed against CMV becomes extremely focussed on just two proteins: UL123 (IE-1) and UL83 (pp65), as early as one week post diagnosis (Khan et al., 2007). An oligoclonal TCR repertoire against CMV epitopes is established early in primary infection by the rapid selection of high avidity public clonotypes (Iancu et al., 2009; Day et al., 2007). With advancing age the CMV specific $CD8^+$ T cell response increases in size (as discussed earlier) and becomes increasingly clonally focussed (Koch et al., 2007). Indeed, lifelong persistent CMV reactivation may drive clonal exhaustion of the most efficient and specific T cells so that an increased number of suboptimal cells are required to control virus infection (Akbar and Fletcher, 2005).

1.4.3.3.4 CMV immunosurveillance amongst the aged

CMV immunosurveillance is widely considered to be maintained even in the very old (Weiskopf et al., 2009). However, CMV may subclinically reactivate with increasing frequency with age (Stowe et al., 2007) and intense immune responses to extracellular CMV in very old subjects have recently been observed, whose magnitude associated with comorbidity and functional decline (Vescovini et al., 2010). Thus, the increased frequency and magnitude of CMV reactivation in advanced age may result in direct or

indirect CMV related pathology, which may significantly contribute towards their impaired health status. It has even been suggested that undiagnosed occult CMV pneumonitis followed by bacterial pneumonia may constitute a common proximate cause of death in the elderly (Pawelec et al., 2010a).

1.4.3.3.5 Effects of CMV on global T cell populations

The effects of continuous lifelong immune surveillance against CMV, as well as the pro-inflammatory and immuno-modulatory effects of the virus, can be associated with global changes to the host's immune profile. Such changes are particularly well documented in the peripheral T lymphoid pool, where CMV is said to leave a fingerprint of infection (van de Berg et al., 2008), being associated with lymphocyte phenotype alterations very similar to those published as age-associated (Weinberger et al., 2007). These changes are most dramatically observed amongst the $CD8^+$ T cell pool, which ages faster than the $CD4^+$ T cell compartment in every aspect studied, even though they principally undergo the same changes (Kovaiou et al., 2005; Weinberger et al., 2007; Czesnikiewicz-Guzik et al., 2008; Koch et al., 2007).

Weinberger et al. observed that all the correlations they described for CMV^+ people could be demonstrated as age-associated in the total study population without reference to CMV status (Weinberger et al., 2007) because of the increasing prevalence of CMV seropositivity with age, such that the majority of elderly donors are CMV-positive (Stowe et al., 2007; McVoy and Adler, 1989; Looney et al., 1999; Dowd et al., 2009). Some investigators have gone so far as to suggest that many of these age-associated changes may be a consequence of increased CMV prevalence

and/or increased duration of infection, rather than ageing *per se* (Pawelec et al., 2004). Therefore, any age-associated correlation that we generate will be stratified by donor CMV status.

1.5 Costimulation and inhibition

1.5.1 Requirement for costimulation

The loss of naïve and accumulation of memory T cells associated with ageing and CMV status is accompanied by concomitant loss of costimulatory molecules as T cells differentiate. Although TCR signalling initiates entry into cell cycle, costimulatory signals are necessary for upregulation of IL-2, IL-2R and increasing cellular metabolism to provide energy for cellular proliferation and acquisition of effector functions without which a tolerogenic response ensues (Fox et al., 2005)

1.5.1.1 CD28

CD28 is well characterised as the most important costimulatory molecule in the primary expansion of antigen specific T cells and although initial findings suggested that CD28 was dispensable for memory T cell responses, more recent data has demonstrated this molecule to be critical for the optimal generation of secondary T cell responses (Boesteanu and Katsikis, 2009)

CD28 binds the homologous B7 family members B7.1 and B7.2 molecules that are both primarily expressed on professional antigen presenting cells (pAPCs). CD28 signalling lowers the TCR activation threshold, enhances production of IL-2 and IL-

2R, promotes cell cycle progression, increases glucose metabolism and upregulates anti-apoptotic proteins (Viola and Lanzavecchia, 1996; Frauwirth et al., 2002; Boise et al., 1995; Seder et al., 1994). The molecular basis of CD28 signalling is further discussed in section 1.5.4.2.

1.5.1.2 ICOS

CD28 is part of a family of five members (the CD28 family) that share structural homology, bind B7 family ligands and function as costimulatory (CD28, ICOS) or inhibitory (CTLA-4, PD-1, BTLA) receptors. ICOS, unlike CD28 is not expressed on naïve T cells but is induced following activation and binds B7h, which is constitutively expressed on B cells and macrophages and can be induced by inflammatory stimuli in non-lymphoid tissue and cells (Yoshinaga et al., 1999). Although ICOS functions overlap with CD28 in early T cell activation, ICOS signalling has emerged as an important player in the selective fine tuning of T cell effector functions (Nurieva et al., 2009). Moreover, ICOS plays a critical role in the generation of optimal humoural immune responses, being key in the generation of Tfh cells and germinal centre reactions (Bossaller et al., 2006; Dong et al., 2001).

1.5.1.3 TNF/TNFR superfamily

A second group of costimulatory molecules have been identified: the TNF superfamily, which are distinguishable from CD28 family members by having a more complex cytoplasmic tail that can recruit the TNF-R associated factor (TRAF) adaptor (Gruss and Dower, 1995). TNF/TNFR ligand pairings with known costimulatory

functions include OX-40/OX-40L, CD27/CD70, 4-1BB/41-BBL, GITR/GITR-L and HVEM/Light.

CD27 is expressed on naïve and central memory but not effector T cells. Its signalling is limited predominantly by restricted expression of its ligand, CD70 to activated T cells, B cells and DCs (Nolte et al., 2009). Multiple studies reveal that CD27 serves as a potent costimulatory molecule with a non-redundant role in the formation of the effector pool by enhancing proliferation and survival of activated T cells (Nolte et al., 2009) and it is further expressed on B cell and natural killer (NK) cells where it can play stimulatory roles.

Other members of the TNFR family, including 4-1BB, CD30, OX40 and HVEM have been proposed to have no unique cellular functions *per se* but that the nature of their distinctive contribution relies upon their expression and that of their ligands (Croft, 2003). Indeed, in this model proposed by Croft et al, HVEM and CD27 play critical roles in initiation of naïve responses alongside CD28 by virtue of their constitutive expression upon naïve T cells. Following naïve T cell activation, HVEM is downregulated whereas CD27 expression increases and OX40 and 4-1BB are induced. Therefore, these receptors govern the T cell response during the later stages with CD27-CD70 interactions driving effector cell expansion, whereas 4-1BB and OX40 may play roles in promoting cell survival with 4-1BB playing a dominant role in $CD8^+$ T cell responses and OX40 governing the $CD4^+$ T cells.

1.5.1.4 Summary

In recent years, an extended array of costimulatory molecules has been identified and characterised. Nevertheless, it is currently unclear to what degree there is functional overlap amongst these pathways and whether a hierarchy in the orchestration of their signals exist. Additional T cell costimulatory molecules are being identified, such as Siglecs -13, -14, -16, and -H, (Varki, 2009) and undiscovered molecules may exist that exhibit yet further diverse and unique functions.

1.5.2 Co-inhibition

1.5.2.1 Introduction

The delivery of a second signal to activate T cells is not just dependent on the presence or absence of costimulatory receptors, as multiple related inhibitory receptors also exist and it is the balance between these inhibitory and costimulatory molecules that is critical to the ultimate fate of cellular responses (Kaufmann and Walker, 2009). The CD28 family members have been crucially implicated in mediating T cell inhibitory (as well as costimulatory) signalling (Chen, 2004). In particular, PD-1 and CTLA-4 are recognised as the primary T cell inhibitory molecules, having critical roles in regulating T cell activation and tolerance (Parry et al., 2005). A multitude of other inhibitory receptors exist, such as the NK family of receptors which can be expressed on effector and memory T cells (further discussed in section 1.5.3.3.1).

1.5.2.2 Function

These co-inhibitory molecules function to prevent inappropriate responses against self. A critical component of peripheral tolerance is the homeostatic migration of tissue

resident DCs to lymphoid organs where they present self-antigens and tolerise autoreactive T cells. Anergy induction is not achieved by the simple absence of costimulatory signals but is dependent on the engagement of CTLA-4 and PD-1 (Abbas and Sharpe, 2005). Moreover, expression of several inhibitory receptors is induced by cellular activation, which limits the extent and severity of the immune response through negative feedback. Similarly, as they are most strongly induced on the most antigen reactive T cells, they prevent initial responses being dominated by a single highly avid clone and thus help maintain the epitope and clonal diversity of immune responses. Nevertheless, exploitation of these negative regulatory pathways represents an immune evasion strategy for several successful pathogens, particularly in facilitating chronic viral persistence.

1.5.3 CTLA-4, PD-1 and KLRG1

1.5.3.1 CTLA-4

1.5.3.1.1 Structure, expression and ligands

Cytotoxic T Lymphocyte Antigen 4 (CTLA-4), also known as CD152, is a dimeric transmembrane glycoprotein with a native molecular weight of 33-37kDa that is a member of the CD28/B7 family. Structurally it consists of an extracellular IgV like domain, a transmembrane domain and a cytoplasmic signalling domain, a design shared with other CD28 family members.

CTLA-4 is specifically expressed on T cells, being constitutively expressed on regulatory T cells, but absent from resting naïve T cells, where it is only induced

following cellular activation, by *de novo* transcription (Sansom and Walker, 2006). Following activation, CTLA-4 is only transiently expressed and rapidly endocytosed such that only small amounts of CTLA-4 can ever be detected on the T cell surface (Rudd et al., 2009). Indeed, the majority of CTLA-4 molecules in resting memory T cells are localised in intracellular vesicles, which following cellular activation, are rapidly translocated to the cell surface where they are expressed in a focussed manner at the immunological synapse (Knieke et al., 2009). Only cell surface expressed CTLA-4 is functional and its level is primarily regulated by the strength of TCR/CD28 stimulation (Valk et al., 2008).

The endogenous ligands for CTLA-4 are the homologous costimulatory molecules B7.1 (CD80) and B7.2 (CD86), which are primarily expressed on activated pAPCs and can be also found on other cells including B and T lymphocytes. CTLA-4 shares these ligands with its related costimulatory molecule CD28 but binds with much greater affinity and avidity possibly compensating for its maximal expression being 30-50 fold less than CD28 (Rudd et al., 2009).

B7.1 and B7.2, despite strong structural similarities, are distinct molecules with unique expression profiles (Bhatia et al., 2006). Indeed, B7.2 is constitutively expressed on pAPCs and is further upregulated after DC activation, peaking after 24 hours, whereas B7.1 is absent from resting pAPCs and is induced at a slower rate, but is stable for a longer time period and peaks after 48-72 hours (Bhatia et al., 2006). Therefore, in part due to their similar expression profiles, B7.2 and B7.1 have been concluded as the predominant CD28 and CTLA-4 ligands, respectively (Bhatia et al.,

2006; Sansom and Walker, 2006). Furthermore, whereas CD28 binds monovalently, CTLA-4 homodimers bind B7.1, though not B7.2, in a divalent manner (Teft et al., 2006) and crystallographic data has shown CTLA-4 interacting with alternating B7.1 homodimers to form a lattice like oligomer that could stereotypically exclude CD28 binding and further compensate for the relative paucity in CTLA-4 expression (Teft et al., 2006). These lattices may also provide appropriate oligomerisation conditions for CTLA-4 signalling to occur, clustering CTLA-4 with TCRs in the immune synapse (Darlington et al., 2005).

1.5.3.1.2 Function

CTLA-4 is an immune regulatory protein whose functional significance is highlighted by the phenotype of CTLA-4 knockout ($^{-/-}$) mice, which exhibit a polyclonal $CD4^+$ dominated lympho-proliferative syndrome, characterised by multi-organ T cell infiltration and death within the first month of life (Waterhouse et al., 1995). The critical importance of CTLA-4 in the proper regulation of human immune responses is underlined by its polymorphisms, with only subtle functional alterations, being associated with cancer susceptibility and autoimmune diseases (Sun et al., 2009).

CTLA-4 has two primary roles in the maintenance of T cell tolerance. It is involved in (a) reducing the pathogenicity of effector T cells and (b) the Treg control of immune responses (Sakaguchi et al., 2009; Friedline et al., 2009; Kolar et al., 2009). CTLA-4 has a general dampening effect on T cell responses by increasing the threshold for effective T cell activation, the net result of which is the inhibition of cytokine production, particularly IL-2 and its receptor, and cell cycle arrest (Verhagen et al.,

2008). However, the precise mechanisms underpinning the functions of CTLA-4 are not fully understood, with several having been proposed but none accounting fully for its inhibitory effects. The most well regarded of these include the preferential binding of B7 ligands to CTLA-4, depriving T cells of critical CD28 costimulatory signals, and CTLA-4 delivering a negative signal via its cytoplasmic domain (Hodi, 2007). The cytoplasmic domain of CTLA-4 neither possesses intrinsic enzymatic activity nor a classic immuno-tyrosine based inhibitory motif (ITIM). However, it does possess a YVKM structural motif that can recruit the phosphatases: SHP-2 and PP2A (Parry et al., 2005), which could oppose the highly organised tyrosine phosphorylation cascade upon which T cell activation is critically dependent (Mustelin and Tasken, 2003). CTLA-4 can also reverse signal via its ligands into DCs where it induces the immunoregulatory enzyme Indoleamine 2,3 dioxygenase (IDO), which may underlie its contribution to Treg function (Onodera et al., 2009; Munn et al., 2004; Fallarino et al., 2003). Alternatively, CTLA-4 may inhibit TCR/CD28 mediated lipid raft expression, protein composition or their signalling microclusters (Rudd et al., 2009). CTLA-4 may also reverse the stop signal, which is transduced by the TCR after it encounters its specific peptide/MHC complex to generate prolonged engagement necessary for T cell activation (Schneider et al., 2006). Overall, it is likely that multiple mechanisms contribute towards CTLA-4 function, with each making distinct contributions in different contexts.

1.5.3.1.3 Therepeutics

Although tumour associated antigens are immunogenic, cancers induce an immuno-regulatory environment that enable them to evade immune detection and suppress

immunogenic responses (de Souza and Bonorino, 2009). Recognition of the critical roles CTLA-4 mediates in the regulation of immune responses has prompted the development of cancer immunotherapies targeting this molecule. Preclinical model data, reviewed in (Weber, 2010) and data from clinical trials (reviewed in (Salama and Hodi, 2011)), support the use of 2 fully human monoclonal CTLA-4 blocking antibodies (temelimulab and ipilimumab) in the induction of durable anti-tumour immunity, best characterised against metastatic melanoma, with one recent randomised trial demonstrating survival benefit amongst patients with advanced melanoma (Hodi et al., 2010). Their use is also associated with a spectrum of immune related adverse events, particularly colitis/diarrhoea, dermatitis, endocrinopathologies and hepatitis, which are commonly mild and manageable, though rare deaths associated with bowel perforation have been reported (Di Giacomo et al., 2010). Conversely, CTLA-4 agonist drugs are also in development for the treatment of autoimmune conditions, with one such therapy (abatacept) approved for use in Rheumatoid Arthritis (Sakthivel, 2009).

1.5.3.1.4 Ageing

Whether CTLA-4 may play a role in T cell ageing has not been comprehensively explored. Higher CTLA-4 mRNA and protein expression (Shimada et al., 2009) have been reported to be expressed in the T cells of old mice but whether this manifests itself as increased surface expression is debated, (Wakikawa et al., 1997; Channappanavar et al., 2009; Shimada et al., 2009). In humans, there is a report of CTLA-4 expression increasing with age on $CD4^+$ T cells (Leng et al., 2002a) and umbilical cord blood lymphocytes expressing less CTLA-4 compared with adult

cohorts (Miller et al., 2002) although no increase in $CD4^+$ Treg CTLA-4 expression with age was observed (Hwang et al., 2009).

1.5.3.2 PD-1

1.5.3.2.1 Structure, expression and ligands

Programmed death -1 (PD-1, CD279) was originally isolated as a protein upregulated in a T cell hybridoma undergoing apoptotic cell death (Ishida et al., 1992) and is a monomeric 55kDa glycoprotein belonging to the CD28 immunoglobulin superfamily of transmembrane proteins (Folkl and Bienzle, 2010). In contrast to the T cell specific expression of CTLA-4, Programmed Death-1 (PD-1) is broadly expressed on lymphocytes, NK cells and macrophages (Freeman et al., 2006). Similarly to CTLA-4, PD-1 is not expressed on resting cells, but is induced only following cellular activation (Freeman et al., 2006), though recent data suggests that PD-1 may be constitutively expressed on naïve T cells at very low levels (Riley, 2009).

PD-1 has 2 endogenous ligands: the structurally related B7 family members PD-L1 (B7-H1; CD274) and PD-L2 (B7-DC; CD273), which consist of an extracellular IgV-IgC domain with short cytoplasmic tails with no known signalling motif, though bidirectional signalling interactions between PD-1 and both PD-L1 and PD-L2 may occur (Keir et al., 2007). PD-L1 is considered its primary ligand by virtue of its broad expression: constitutively on DCs, B cells, macrophages and T cells and is further upregulated upon activation and can also be induced on parenchymal cells in lymphoid and non-lymphoid tissues (Freeman et al., 2006), whereas PD-L2 is exclusively and inducibly expressed on DCs and monocytes (Freeman et al., 2006).

Indeed, IFNs are powerful inducers of PD-L1 expression on APCs, endothelial and epithelial cells such that during inflammatory immune responses PD-L1 expression is extensive and intense (Freeman et al., 2006).

1.5.3.2.2 Physiological function

PD-1 is an immune inhibitory molecule that is critically involved in several physiological processes, including the central induction and peripheral maintenance of tolerance to both self-antigens and in preventing immunopathological responses to chronic pathogens, limiting the extent and duration of adaptive responses and in the maintenance of tolerance at immuno-privileged sites. Its functional significance is highlighted by the phenotype of PD-1 genetically deficient mice which develop a spontaneous tissue specific autoimmunity, whose manifestation depends on the genetic background of the mouse, and it also exacerbates disease in other mouse models of autoimmunity and graft rejection (Folkl and Bienzle, 2010; Okazaki and Honjo, 2007). Moreover, human PD-1 polymorphisms have been associated with susceptibility to a wide range of autoimmune diseases (Okazaki and Wang, 2005).

The functions of PD-1 fall into two main categories:

1) Induction and maintenance of tolerance to self-antigens

Whereas a role in central tolerance is debated, PD-1/L clearly has a critical role in the regulation of peripheral tolerance (Folkl and Bienzle, 2010). Both CTLA-4 and PD-1 interactions play critical and non-redundant roles in the induction of homeostatic peripheral tolerance mediated by DCs (Probst et al., 2005). However, unlike CTLA-4,

PD-1 interactions are also critical in their peripheral maintenance, a consequence of PD-L1 expression on non-haematopoietic cells regulating effector T cell responses in the periphery (Keir et al., 2006). An extreme example of this is observed in immune privileged sites, which are able to tolerate introduction of immunogenic antigens without eliciting an inflammatory immune response, where PD-1/L1 interactions make critical contributions (Usui et al., 2008; Cheng et al., 2009). PD-1/L interactions also play a role in materno-fetal tolerance (Wafula et al., 2009).

2) Limitation of immunopathologic responses directed against chronic pathogens: Infections by acute pathogens result in a transient induction of PD-1 expression on T cells, which rapidly returns to basal levels following antigen clearance, giving rise to highly functional memory cells (Barber et al., 2006). However, following persistent pathogen infection, antigen is not cleared and PD-1 expression remains high and dysfunctional or exhausted specific memory cells arise (Barber et al., 2006). This may have evolved as a host defence mechanism to limit potentially detrimental inflammatory consequences of immune responses directed against relatively innocuous persistent pathogens. Indeed, PD-L1$^{-/-}$ mice infected with a chronic lymphocytic chorio-meningitis virus (LCMV) strain die as a result of immuno-pathological damage, whereas those infected with an acute self-limiting strain behaved as wild type (WT) mice (Barber et al., 2006). Adenovirus infected PD-1$^{-/-}$ mice clear the virus but suffer a severe hepato-cellular injury compared with WT mice (Okazaki and Honjo, 2006). Indeed, there is an idea that there is a limited period of time to eliminate an immunogenic antigen, otherwise it can be assumed to be either self or from a persistent pathogen, and the PD-1/L1 axis comes to dominate and

suppress an otherwise immunopathic response. The oscillating immune response to Hepatitis B Virus (HBV) infection in association with PD-1 may support such a scenario (Isogawa et al., 2005).

1.5.3.2.3 Exploitation by chronic pathogens

Many highly pathogenic persistent pathogens take advantage of the PD-1/L pathway to facilitate their persistence in the host. HIV specific $CD8^+$ T cells highly express PD-1 with its expression correlating with the extent of HIV specific $CD8^+$ T cell dysfunction and markers of disease progression. Moreover, blocking PD-1/L1 interactions enhanced their proliferative and effector responses (Trautmann et al., 2006; Kaufmann and Walker, 2009; Day et al., 2006; Petrovas et al., 2006), though such findings have been disputed (Sauce et al., 2007a). Similarly, PD-1 is critically implicated in $CD8^+$ T cell dysfunction specific for persistent viral infections including HBV (Boni et al., 2007), HCV (Golden-Mason et al., 2007; Urbani et al., 2006), human T lymphotophic virus-1 (HTLV-1) (Kozako et al., 2009), Simian Immunodeficiency Virus (SIV) (Velu et al., 2009) and LCMV (Barber et al., 2006). In addition, PD-L1 is also upregulated on APCs during many persistent infections (Kaufmann and Walker, 2009), which further enhances PD-1 signalling on virus specific T cells and DCs expressing PD-L1 display a 'paralysed' phenotype, (as described in section 1.4.1.5). Indeed, PD-L1 expression is a surrogate marker of HIV disease progression (Trabattoni et al., 2003). Moreover, PD-1 blockade *in vivo,* using a Rhesus macaque model of SIV infection, revealed significant reductions in plasma viral load and improved survival, alongside enhancement of cellular and humoural immunity, both in the blood and tissues, with no observable toxicity (Velu et al.,

2009). Therefore, manipulation of the PD-1/L pathway may show promise in the treatment of HIV/AIDS and other chronic pathogenic infections.

1.5.3.2.4 Cellular functions

The major effect of PD-1 signalling was initially thought to be on proliferation (Freeman et al., 2006) but more recent data emphasise its role in promoting cell death (Petrovas et al., 2006; Muhlbauer et al., 2006). Nevertheless, the precise outcome of PD-1 signalling may depend upon the type of APC and micro-environment (Carter et al., 2002; Yamazaki et al., 2005).

1.5.3.2.5 Ageing

There are reports of uninfected old, but not young, mice highly expressing PD-1 (mRNA and protein) on their T cells (Shimatani et al., 2009; Channappanavar et al., 2009; Shimada et al., 2009) and PD-1/L blockade augmenting the virus specific responses of aged mice (Suvas et al., 2007; Mirza et al., 2010). However, others have made contrasting observations (Lages et al., 2010)**,** suggesting PD-1 may be differentially regulated in different mouse strains. Moreover, several reports suggest PD-1 expression does not vary with age on human T cells (Fann et al., 2005; Czesnikiewicz-Guzik et al., 2008; Henson et al., 2009). Nevertheless, PD-1 may play a role in driving immunosenescence by virtue of its expression on CMV specific $CD8^+$ T cells, (Petrovas et al., 2006; Day et al., 2006; Sauce et al., 2007a; Trautmann et al., 2006), thereby contributing towards their progressive dysfunction, clonal expansion and CMV reactivation with age, which represents a key immuno-senescence driving force (as discussed earlier).

1.5.3.3 KLRG1

1.5.3.3.1 NK cell receptors on T cells

NK cell receptors (NKRs) can accumulate on late stage differentiated T cells, particularly in the $CD8^+$ T cell compartment, where they exhibit a distinct pattern of NKR expression from that expressed by NK cells (Robbins et al., 2003; Abedin et al., 2005; Boucher et al., 1998; Pawelec, 2009; Vivier and Anfossi, 2004). Moreover, their expression increases with age such that the $CD8^+$ TCR repertoire in old age consists of TCR clonal but NKR diverse cells (Abedin et al., 2005; Boucher et al., 1998; Uhrberg et al., 2001; Vallejo, 2006).

Ligation of NKRs on T cells only transduces signals if TCR is coligated and functions to alter the threshold of T cell activation (Fasth et al., 2010; Vivier and Anfossi, 2004). Their functional significance on T cells is highlighted by their dysregulated expression being associated with several types of chronic inflammatory diseases (Fasth et al., 2010; Chen et al., 2009; Meresse et al., 2006), persistent pathogens (De et al., 1997; Berg et al., 2003) and tumours (Vivier and Anfossi, 2004).

The expression of NKRs on T cells has been suggested to have several physiological roles. Inhibitory NKR expression may promote peripheral tolerance, being induced following repeated stimulation and causing inhibition of autoreactive responses. Alternatively, inhibitory NKRs exert a strong impairment on T cell proliferation and a leaky impairment on effector functions (Arlettaz et al., 2004), consistent with a role in protecting chronically antigen-exposed T cells from exhaustive expansion.

Additionally, activatory NKR can also be expressed on T cells (Fasth et al., 2010; Monsivais-Urenda et al., 2010), which may represent a secondary immune diversification that compensates for the TCR repertoire and CD28/CD27 expression loss with age (Vallejo, 2006).

1.5.3.3.2 Structure, expression and ligands

Killer cell lectin-like receptor subfamily G member 1 (KLRG1) is a type 1 transmembrane inhibitory receptor belonging to the C-type lectin like superfamily that was first identified in a rat basophilic leukaemia cell line and originally termed MAFA (Mast cell function associated antigen) (Voehringer et al., 2001b). KLRG1 is expressed on the cell surface as a monomer or a disulphide linked homodimer at comparable levels (Rosshart et al., 2008). Additionally, a substantial fraction exists as disulphide linked trimeric and tetrameric complexes and can non-covalently associate to form even larger multimeric forms (Rosshart et al., 2008). This multimerisation increases KLRG1 avidity and helps compensate for the very low affinity for its ligand compared with other inhibitory NKRs (Li et al., 2009).

KLRG1 is expressed on 50-80% of NK cells and antigen experienced αβ T cells (around 20% of $CD4^+$s and 40% of $CD8^+$s in a young adult) (Voehringer et al., 2002) and is also expressed on γδ (Eberl et al., 2005) and regulatory T cells (Banh et al., 2009). KLRG1 is highly expressed on mouse T and NK cells following infections (Banh et al., 2009) and human $CD8^+$ T cells specific for persistent but not acute pathogens (Rosshart et al., 2008) and increases with human age and T cell differentiation (Voehringer et al., 2002; Thimme et al., 2005; Ouyang et al., 2003a;

Ito et al., 2006). This suggests that repetitive and persistent antigen stimulation is the factor driving KLRG1 expression and is consistent with a stochastic model of inhibitory NKR induction on T cells after antigenic stimulation proposed by Vivier and Anfossi (Vivier and Anfossi, 2004). Nevertheless, other factors must also be required as repeated T cell activation *in vitro* is insufficient to induce KLRG1 expression (Voehringer et al., 2002), and indeed, no known *in vitro* stimulation upregulates KLRG1 and its expression is downregulated and lost within 72 hours *in vitro* (Voehringer et al., 2002).

KLRG1's physiological ligands are the classical cadherin family members Epithelial- (E-), Neural (N-) and Retinal (R-) cadherins (Ito et al., 2006). These are a family of ubiquitously expressed transmembrane glycoproteins linked to the cytoskeleton that mediate homophilic adhesion to form tight adherent junctions between adjacent cells (Banh et al., 2009).

Aside from KLRG1, E-cadherin can also bind the integrin $\alpha_E\beta_7$ (Cepek et al., 1994). Integrins are heterodimeric transmembrane proteins that function as cell adhesion and accessory molecules during T cell stimulation (Banh et al., 2009). $\alpha_E\beta_7$ is highly expressed on intra-epithelial lymphocytes, particularly $CD8^+$ T cells, where it mediates adhesion to epithelial cells and enhances T cell function (Agace et al., 2000), providing immuno-surveillance against damaged, infected or transformed epithelial cells.

1.5.3.3.3 Cellular effects

KLRG1 expression identifies $CD8^+$ T cells that can perform immediate effector functions but exhibit severely impaired proliferative capacity (with KLRG1 expression being a better marker of $CD8^+$ T cell proliferative inability than CD28 loss (Voehringer et al., 2002)) and may thus represent a highly differentiated replicatively senescent population (Voehringer et al., 2001a; Voehringer et al., 2002). This proliferative defect is observed even in the presence of exogenous IL-2 (Heffner and Fearon, 2007), distinguishing T cells expressing KLRG1 from the exhausted phenotype mediated by PD-1 signalling (Carter et al., 2002). Moreover, rather than just being a marker, a direct role for KLRG1 as an inhibitory receptor has been demonstrated in murine and human NK and T cells (Rosshart et al., 2008; Schwartzkopff et al., 2007).

1.5.3.3.4 Physiological function

Aside from roles in preventing persistently stimulated T cells from exhaustive expansion (as discussed in section 1.5.3.3.1), KLRG1's physiological function may lie in the maintenance of peripheral tolerance, preventing NK and T cell mediated destruction of self tissues expressing E, N or R cadherins. Conversely a role for KLRG1 in tumour immunosurveillance can be suggested, analogous to missing self-recognition by inhibitory NKRs that bind MHC class I (Schwartzkopff et al., 2007; Colonna, 2006). Indeed, the mutation or downregulation of E–cadherin is a common feature in tumour development that results in the loss of cell contact inhibition and acquisition of cellular motility that facilitates tumour metastases (Makrilia et al., 2009) but this also results in loss of inhibitory KLRG1 interactions that render tumour cells a target for NK and $CD8^+$ T cell mediated killing.

Nevertheless, E-cadherin exists as strand exchanged homodimeric complexes that constitute adherent junctions on the basolateral membrane of adjacent epithelial cells and thus is not easily accessible to NK or T cells (Nakamura et al., 2009). Moreover, KLRG1 can only recognise the monomeric form of E-cadherin, which is thought to be expressed solely on abnormal epithelial tissues (Nakamura et al., 2009). However, E-cadherin can also bind the integrin $\alpha_E\beta7$, which is highly expressed on intraepithelial T cells and whose ligation promotes T cell activation (see 1.5.3.3.2). Therefore, when epithelial cells are subject to transformation, viral infection or mechanical injury, the tight junctions between cells become disrupted resulting in the expression of monomeric E-cadherin that renders them a target for intraepithelial T cell killing via $\alpha_E\beta_7$ ligation.

Langerhans cells (LCs), a DC subset that reside in the skin, form networks anchored to neighbouring keratinocytes through homotypic binding of E-cadherin (Fu and Jiang, 2010). However, under steady state conditions, LCs migrate into the cutaneous lymph nodes, present self antigens and induce tolerance to any autoreactive T cells encountered. The generation, regulation and function of such tolerogenic DCs remain poorly understood though they have been frequently described or predicted as immature. However, immature DCs neither efficiently present antigen nor effectively interact with T cells (Fu and Jiang, 2010). Nevertheless, disruption of E-cadherin mediated interactions results in the alternative maturation of DCs into cells that migrate into secondary lymphoid organs but promote tolerogenic rather than immunogenic T cell responses (Jiang et al., 2007). Furthermore, KLRG1 has recently

been identified as capable of reverse signalling into E-cadherin expressing DCs and exerting immuno-suppressive effects by inhibiting cadherin dependent cellular adhesion and inhibiting their ability to release inflammatory cytokines (Banh et al., 2009). Moreover, E-cadherin expression is not limited to LCs but is expressed on a wide variety of APCs (Henson et al., 2009). Therefore, ligation of E-cadherin by KLRG1 may mediate the steady state migration of DCs to secondary lymphoid organs where they tolerise autoreactive T cells.

1.5.3.3.5 Ageing

KLRG1 expression broadly correlates with age-associated T cell dysfunction, being upregulated with age on $CD8^+$ (Ouyang et al., 2003a; Thimme et al., 2005) and to a lesser extent on $CD4^+$ T cells (Koch et al., 2007), showing increased expression on CMV^+ individuals and being expressed on the vast majority of CMV specific cells which further increases with age (Vasto et al., 2007). Moreover, KLRG1 appears to act as a marker of end-stage differentiation and has been suggested to play a role in driving T cell clonal exhaustion (Voehringer et al., 2002) and thus may contribute towards age onset T cell immuno-dysfunction.

1.5.4 Molecular Basis of T cell Activation and Inhibition

1.5.4.1 TCR Signalling

Cross linking TCRs *in vitro* can activate T cells, suggesting a similar mechanism of activation to B cell receptors (BCRs), whose clustering by a multivalent antigen activates B cells *in vivo* (Tolar et al., 2008). However, as single antigenic peptides-MHC complexes can trigger cytolytic activity in $CD8^+$ T cells (Sykulev et al., 1996),

several hypotheses of T cell activation independent of TCR clustering have arisen. These include antigen binding inducing conformational changes in the TCR or its signalling complex (Ma and Finkel, 2010) that either generates an activatory signal itself or produces a stop signal that prolongs TCR-APC engagement, allowing the formation of an immunological synapse that concentrates, enhances and sustains signalling (Dustin, 2009). Other TCR activation proposals do involve clustering, for example the pseudo-dimeric (Krogsgaard et al., 2005) and permissive geometry models (Minguet and Schamel, 2008).

TCR heterodimers are associated with a signalling complex consisting of 4 CD3 chains (2 CD3ε, 1 CD3δ and 1 CD3γ) and a ζ homodimer that together give the TCR complex a total of 10 immuno-tyrosine based activation motifs (ITAMs). Upon TCR engagement, the ITAM tyrosine residues become phosphorylated by the kinases Lck and Fyn, associated with the CD4/CD8 and CD3 chains respectively. These are normally kept inactive by inhibitory phosphorylation mediated by Csk, which is anchored to lipid rafts by the transmembrane adaptor PAG. Following TCR ligation, PAG is dephosphorylated and releases Csk which relieves the inhibition of Lck and Fyn.

Phosphorylated ITAM tyrosine residues serve as a binding site for the two SH2 domains of ZAP-70, a tyrosine kinase which, once recruited, become phosphorylated and activated by Lck, and goes on to phosphorylate the scaffold proteins: the transmembrane LAT and the cytosolic SLP-76. Together in a complex with Gads, these recruit and activate Phospholipase C-γ (PLC-γ), a key signalling molecule that

catalyses breakdown of the membrane lipid PIP2 into cytosolic IP3 and the membrane lipid DAG. Hereafter, the TCR signalling pathway splits into the following three branches, each of which results in the activation of a different transcription factor.

i) IP3 binds receptors on the endoplasmic reticulum where it induces cytosolic Ca^{2+} release that binds calmodulin enabling it to activate calcineurin that dephosphorylates and activates the transcription factor NFAT.

ii) DAG diffuses in the plasmamembrane activating several proteins including PKC and RasGRP, which is a GTP exchange factor that activates the small G protein Ras. This in turn triggers the MAP kinase cascade that promotes the formation of the transcriptional regulator AP-1.

iii) The membrane recruitment and activation of PKC results in it forming a membrane associated complex that recruits and activates the IκK kinase complex which phosphorylates and targets IκB for degradation. NF-κB is normally held in an inactive state bound to IκB, and once released, NF-κB enters the nucleus and functions as a transcription factor.

1.5.4.2 CD28

TCR engagement activates the transcription factors AP-1, NF-κB and NFAT that induces a genetic program that can result in either a tolerogenic T cell response or an immunogenic one (Riley et al., 2002). The choice between these T cell fates is largely determined by costimulatory signals. CD28 is considered the quintessential

costimulatory molecule that can rescue naïve T cells receiving a TCR stimulus alone from anergy/apoptosis and initiate an immune response. Indeed, its functional significance is highlighted by the severe immuno-deficiencies of $CD28^{-/-}$ mice (Shahinian et al., 1993).

Ligation-dependent tyrosine phosphorylation of CD28 results in the recruitment and activation of PI3K. PI3K phosphorylates phosphatidylinositol 4,5-bisphosphate (PIP2) to phosphatidylinositol 3,4,5-trisphosphate (PIP3), which binds PH domain containing proteins to the membrane, including the serine/threonine kinase Akt (Frauwirth et al., 2002). Akt is thought to play an important role in diverse cellular processes including telomerase activation, survival, cytokine synthesis and glucose metabolism (Parry et al., 2005). However, CD28 activity independent of PI3K/Akt has been demonstrated (Sanchez-Lockhart et al., 2004) suggesting another distinct pathway is critical for CD28 costimulatory activity. Such a pathway may be provided by Grb2 family adaptor proteins Grb2 and Gads, which are also recruited by phosphorylated YVKM motifs at CD28 and augment TCR induced PLC-γ and Vav activation (Watanabe et al., 2006). Alternatively, further downstream of the YVKM motif, CD28 has two proline rich regions that have been reported to bind Src and Tek kinases (Rudd et al., 2009), which could further enhance T cell activation.

CD28 does not stimulate identical signalling effectors as TCR ligation, but primarily provides a potent synergistic induction of NFAT, NF-κB and AP-1 (Acuto and Michel, 2003). TCR ligation induces the expression of over 3000 genes and the genes modified by CD28 expression appear virtually identical (Diehn et al., 2002; Riley et

al., 2002). This suggests that CD28 functions mainly as an amplifying mechanism to overcome signalling thresholds that TCR signalling alone cannot reach in a physiological setting. However, CD28 also has a major impact on chromatin remodelling and can downregulate a significant proportion of these TCR induced genes (Diehn et al., 2002; Riley et al., 2002), indicating that CD28 mediated signalling is not merely repetitive of TCR but also alters the balance of signalling events and feedback circuits. Indeed, it may be that modulation of large sets of identical TCR-induced genes represent a common mechanism of costimulatory and coinhibitory molecules; ICOS induces and CTLA-4 counter-regulates virtually the same genes that were induced or repressed by CD28 (Riley et al., 2002). CD28 can also affect gene regulation independently of TCR ligation, which may have contributed towards the disastrous auto-immune complications in the trial of TGN1412 (Farzaneh et al., 2007).

1.5.4.3 Inhibitory receptors

Inhibitory receptors only modulate T cell functions when co-engaged with the TCR (Greenwald et al., 2005; Rosshart et al., 2008), which results in the phosphorylation of ITIM (for KLRG1 and PD-1) or YVKM (in the case of CTLA-4) motifs by Src family kinases, and the recruitment of Sh2 domain-containing phosphatases. These phosphatases antagonise the signal transduction processes of T cell and costimulatory receptors, which are initiated and sustained by the actions of protein kinases (as described earlier). Four such phosphatases have been identified: the two tyrosine phosphatases SHP-1 and SHP-2, and the lipid phosphatases SHIP-1 and -2 (Daeron et

al., 2008) and their specificity for different ITIMs is determined by two symmetrical hydrophobic residues at positions Y-2 and Y+2 (Daeron et al., 2008).

The inositol phosphatase, SHIP-1, recruited by KLRG1 but not CTLA-4 or PD-1, mediates its inhibitory effect through hydolysing PIP3 (Daeron et al., 2008), which is responsible for the recruitment of critical T cell activation signalling molecules containing the PH domain, such as Akt and PLC-γ. In contrast, the tyrosine phosphatases: SHP-1 and SHP-2 could theoretically dephosphorylate any of the phosphorylated proteins co-localised in the immune synapse, thereby attenuating both proximal and distal events in the T cell activation cascade (Daeron et al., 2008).

CTLA-4, PD-1 and KLRG1 have all been demonstrated to recruit at least one of these tyrosine phosphatases (CTLA-4: SHP-2, KLRG1: SHP-2 and PD-1: SHP-1 and SHP-2 and thereby attenuate TCR/CD28 mediated signalling and increase the activation threshold (as described in section 1.5.3). However, KLRG1 also recruits SHIP and can thus inhibit Akt activity. Moreover, CTLA-4 can recruit the serine/threonine phosphatase PP2A and has been demonstrated to inhibit Akt in a PP2A dependent manner (Parry et al., 2005). PD-1 inhibits Akt via the dephosphorylation and inactivation of PI3K (Parry et al., 2005). This contrasts with KLRG1 and CTLA-4 which inhibit Akt in a manner that preserves PI3K activity, thereby allowing expression of certain pro-survival genes such as Bcl-xl (Riley, 2009). This selective inhibition of Bcl-xl and several other growth factors may at least partially account for PD-1 signalling effects on promoting cell death (as discussed in section 1.5.3.2.4). Moreover, this may provide a molecular explanation for why IL-2 signalling can

override the effects of PD-1, but not KLRG1 or CTLA-4 ligation (Carter et al., 2002), as IL-2 signalling triggers Akt activity in a PI3K independent manner (Lockyer et al., 2007). Nevertheless, the precise signalling pathways of CTLA-4 (see Fig 1.02), PD-1 (see Fig 1.03) and KLRG1 (see Fig 1.04) remain poorly defined, though a major contribution to their distinct functions resides in their differing expression patterns, ligands and kinetics. Additionally, inhibitory receptors may also regulate the gene expression profile of T cells. Indeed, PD-1 has recently been reported to induce the expression of a series of proteins that, in combination with its inhibition of TCR/CD28 signalling, may mediate T cell exhaustion (Quigley et al., 2010).

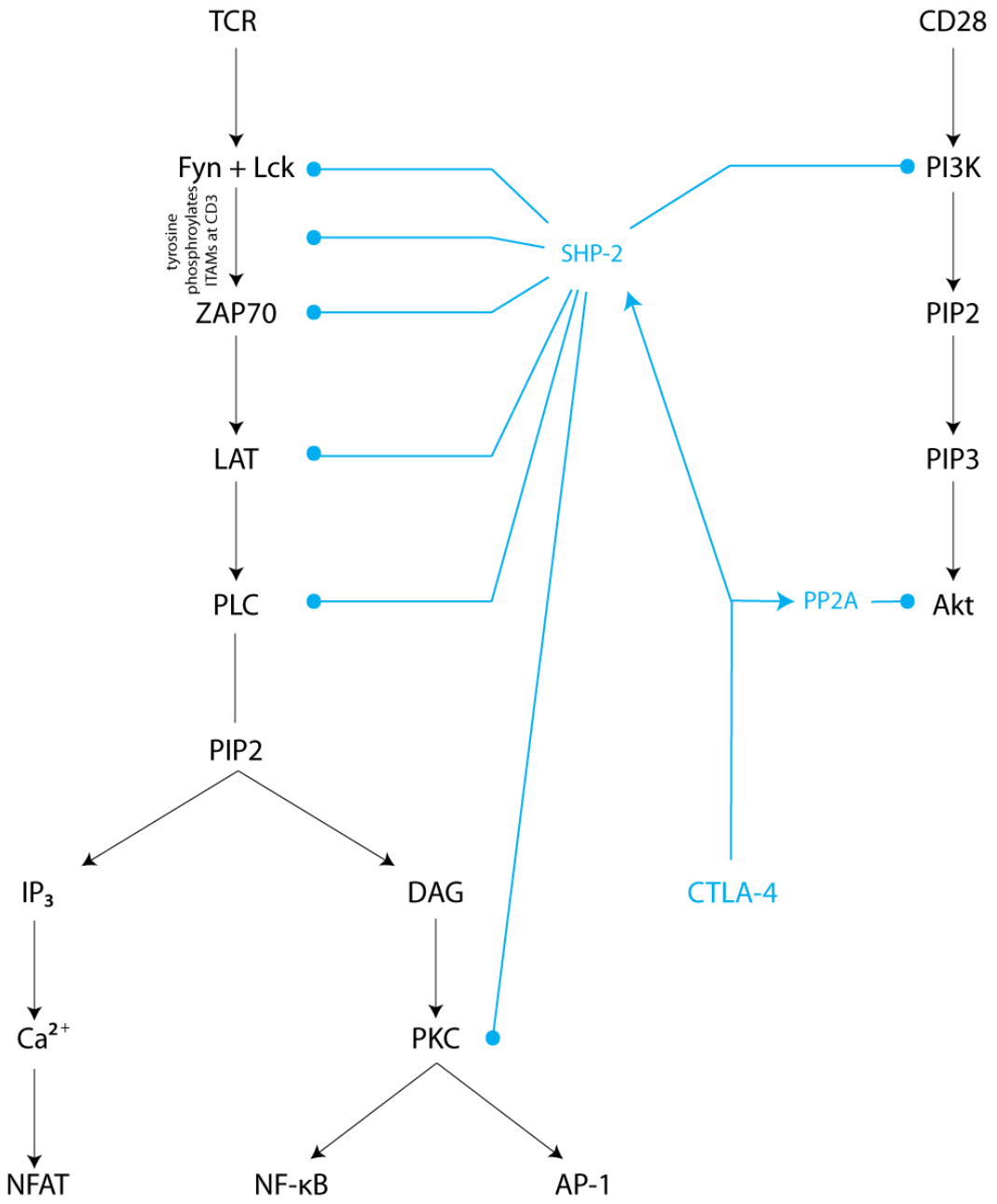


Fig 1.02 Mechanism by which CTLA-4 mediates inhibition of TCR and CD28 signalling

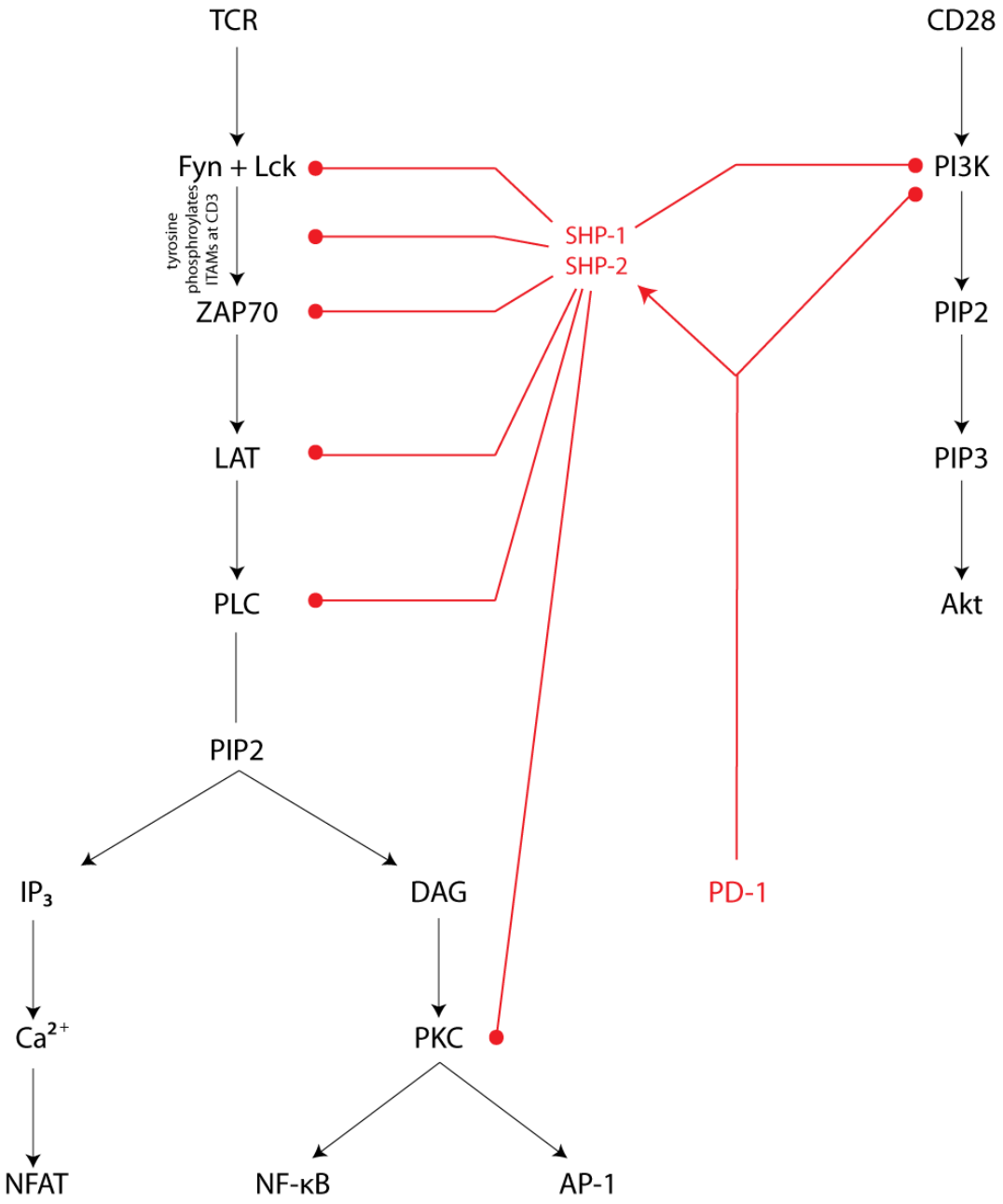


Fig 1.03 Intracellular signalling pathways of PD-1

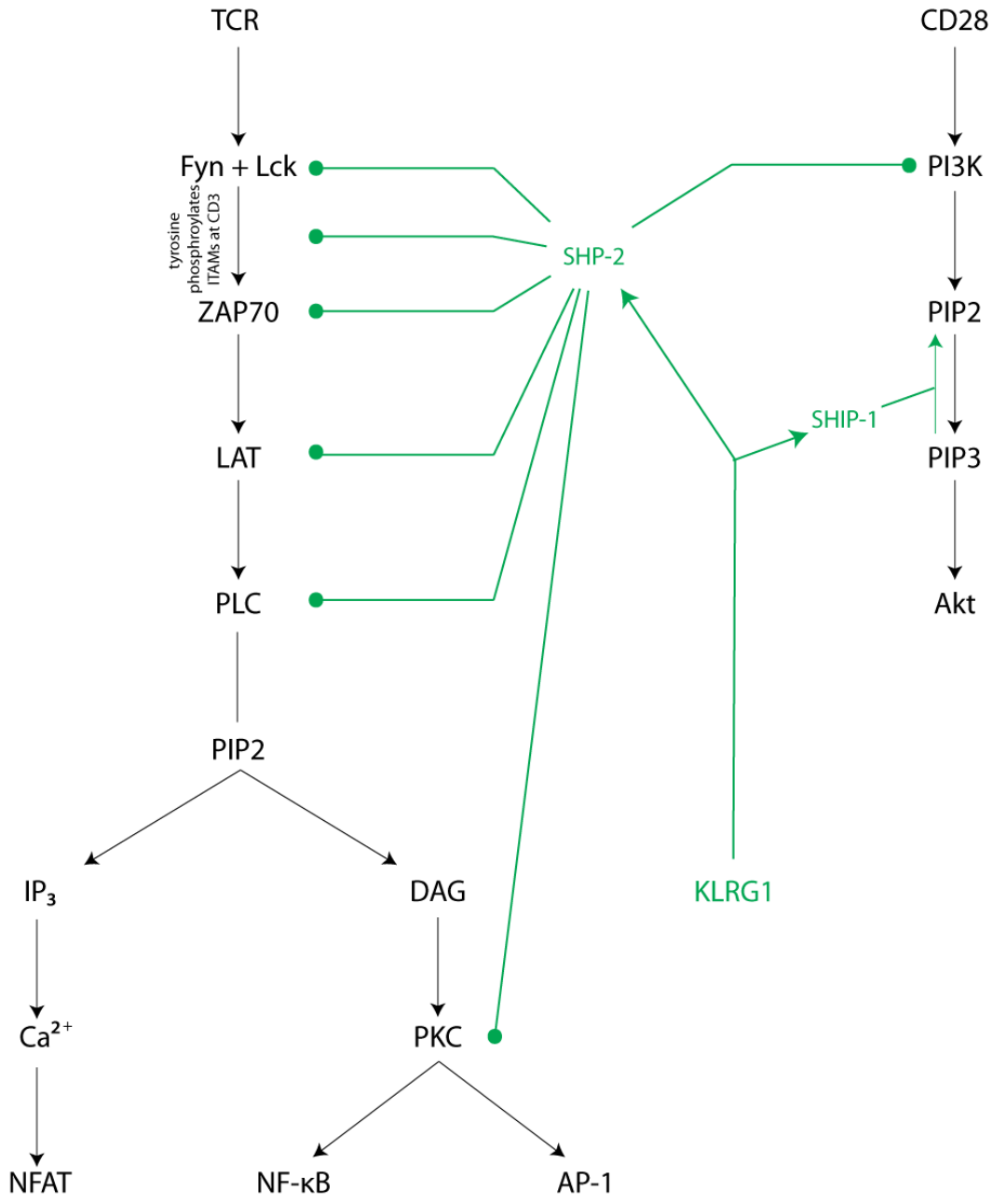


Fig 1.04 Molecular mechanisms of KLRG1 mediated T cell inhibition

1.6 Models for Studying Human Ageing

One of the major difficulties in the study of human ageing is its duration, developing over several decades, thus making *in vivo* longitudinal studies highly impractical.

1.6.1 Animal Models

One solution is to study model organisms which have much shorter lifespans (2-4 years for most laboratory rodents). The almost ubiquitous nature of aging across the animal kingdom is suggestive of common ageing pathways operating across many phyla (Longo and Fabrizio, 2002). Moreover, the ability to control and manipulate their environment and genetics as well ease of access to tissue samples, make animal models a potentially highly useful tool in the study of human ageing.

1.6.1.1 Impracticalities in the Mouse Model

The most heavily utilised mammalian model of human ageing is the mouse, due to their short life cycles and small size. However, evidence is accumulating that such animals are poor models of human ageing. For example, some mice exhibit causes of ageing unrelated to those of humans, such as the Australian species *Antechinus stuartii*, whose males become intoxicated with sex hormones during the mating season that increases their libido to the point that they do not eat and eventually die of 'sexual stress' (Holleley et al., 2006). Moreover, even amongst mice that age gradually in a manner analogous to humans, such as *Mus musculus* (the most commonly used laboratory mouse), they exhibit several critical differences that render

mice a poor model for human immune ageing (Akbar et al., 2000; Gordon et al., 2001). These include: -

1) Differences in telomere regulation.

Telomere attrition has been suggested as a major player in driving the human ageing process (as discussed in section 1.2.3.2.3). However, human telomere lengths are much shorter (12-16kB) than those in mice (90-100kB) (Akbar et al., 2000), especially considering the much greater lifespan of humans. Moreover, the enzyme telomerase, which maintains telomere lengths, is absent in most normal human somatic cells, but present in most mice cells (Blasco, 2005). Furthermore, human telomerase dysfunction exhibits itself as a premature ageing syndrome (Dyskeratosis congenita, DKC), whereas telomerase-deficient mice are normal for up to 6 generations (Blasco, 2005). In summary, the attrition of telomere lengths is a critical determinant of human aging, but plays a comparatively minor role in mouse aging.

2) Loss of CD28 expression on $CD8^+$ T cells.

This is considered one of the most consistent biomarkers of human immune system ageing and constitutes an important parameter of the Immune Risk Profile (as described in section 1.4.2). In contrast, murine $CD8^+$ T cells maintain CD28 expression with age (Engwerda et al., 1994).

3) Other contributing factors

Other factors undermining the use of laboratory mice as a model for human ageing include their vastly different immunological history, as antigenic load is considered a

critical factor driving age-associated immune changes (as described in section 1.3.2) In addition, the comparatively reduced lifespan of mice suggest divergent maintenance and repair systems, which play major roles in driving aging. For example, loss of telomerase is an important anti-cancer mechanism in human cells whilst its maintenance in murine cells may reflect cancer being a less severe mouse mortality factor (Wright and Shay, 2000). Finally, mice developed for laboratory research have been selected for high fertility and thus have been subject to different evolutionary pressures than humans.

1.6.1.2 Longitudinal studies

A compromise to the impracticality in performing human longitudinal studies is to limit the study to the very elderly (octo- or nona-genarians), as there will be sufficient mortality to enable meaningful associations within a time frame of several years. This was the approach taken by the Swedish OCTO and NONA studies from which the Immune Risk Phenotype was defined. Nevertheless, even this approach carries some significant limitations, notably that such populations are already successfully aged who may possess a confounding genetic or environmental factor that limits their applicability.

1.6.1.3 Cross sectional studies

As longitudinal studies are challenging in humans, most population studies examining human ageing have utilised a cross-sectional approach comparing young and old individuals. However, the conditions applied during the life course of the current elderly differ to the younger generations. Such conditions, including nutrition,

pathogen load, environment and population genetics (Caruso et al., 2009), may act as confounding variables that account for some of the published differences between the old and young, rather than the process of ageing *per se*. Despite these limitations, we utilised cross sectional studies in our investigations, as this was the only practical way to perform our studies in humans and believe our results to represent this topic as accurately as possible.

1.7 Aims

Ageing impinges upon the immune system, particularly the T cell compartment, and lifelong antigenic burden is increasingly implicated in this decline. Various persistent antigenic challenges are associated with dysfunctional T cells and upregulation of inhibitory receptors. Blockade of such receptors has been shown to reinvigorate cellular responses. This thesis investigates the hypothesis that inhibitory receptor expression increases with age and/or CMV status, which may contribute towards the characteristic dysfunctions of aged T cells and define a reversible defect.

The specific aims of this study were to: -

1) Investigate whether inhibitory receptor blockade can functionally reinvigorate aged T cells.
2) Assess whether different inhibitory receptors are preferentially utilised by T cells at distinct differentiation stages.

3) Define the role of these inhibitory receptors in mediating the dysregulated immune response directed against CMV.
4) Determine how the age related lymphocyte telomere attrition and impaired telomerase induction are influenced by CMV infection and inhibitory receptor expression.
5) Characterise the factors driving the variations of inhibitory receptor expression with donor CMV status and age.

2 Materials and Methods

2.1 Peripheral blood cell source, culture, sorting and storage

2.1.1 Volunteer Recruitment

Donor samples were obtained in accordance with the ethical committee of Royal Free and University College Medical School and voluntary informed consent was obtained in accordance with the Declaration of Helsinki. Heparinised peripheral blood samples were taken from healthy volunteers. Old donors did not have any co-morbidity, were not on any immunosuppressive drugs and retained physical mobility and lifestyle independence. Where the data is stratified by age, young is defined as individuals between 20-35 years (median age 30) and old subjects over 65 years (median age 78).

2.1.2 Separation of peripheral blood mononuclear cells from whole blood

Heparinised venous blood was mixed in a 1:1 ratio with Hanks Balanced Salt Solution (HBSS) (Invitrogen, Paisley, UK) and layered onto Ficoll-Hypaque (Amersham Biosciences, Buckinghamshire, UK) in 50ml Falcon tubes before centrifugation at 800×g for 20 minutes with no brake. The buffy coat at the interphase layer was harvested and washed twice for 10 minutes in excess HBSS by centrifugation firstly at 650×g and then at 350×g.

2.1.3 Viable cell counts

Cell suspensions were diluted 1:1 with 0.4% trypan blue (Sigma-Aldrich, Dorset, UK) and incubated for 1 minute at room temperature before 8µl were inserted beneath the coverslip on an improved Neubauer chamber (Weber Scientific International, Sussex, UK) and the cells were counted. Dead cells were stained blue and were therefore excluded from the count. The number of cells in the central grid that excluded dye were enumerated and this number was multiplied by the (dilution factor x 10,000) to give the number of cells per ml.

2.1.4 Cell culture

Cell preparation was carried out using RPMI 1640 medium (GIBCO, Invitrogen, Paisley, UK) supplemented with GPS (100U/ml penicillin, 100µg/ml streptomycin, 2mM L-glutamine), which will be referred to as RPMI hereafter. This medium was supplemented with 10% heat-inactivated Foetal Calf Serum (FCS) or pooled human AB serum (all from Sigma-Aldrich, Dorset, UK) for cell culture. Non-sterile solutions were filtered through 0.22µm filters and all tissue culture plastics, tubes and filters were purchased from BD Biosciences, (Oxford, UK)

2.1.5 Cell storage

PBMCs were pelleted (650×g, 5 mins) and supernatant decanted. Working quickly, cells were resuspended in FCS + 10% DMSO and aliquoted into cryovials at 5 x

10^6cells / vial and placed in a Mr. Frosty freezing chamber (Fisher scientific, Leicestershire, UK) and put into a –80°C freezer.

2.1.6 Frozen cell retrieval

Working quickly, PBMCs were thawed using pre-warmed complete media +10% FCS then pelleted (650×g, 5 minutes) and resuspended in RPMI +10% FCS and counted.

2.1.7 Cell Sorting

Magnetic Cell Sorting (MACS, Miltenyi Biotec, Surrey, UK) allows the purification of cell subsets from complex cell mixtures. Cells can be specifically labelled using MACS microbeads, which are extremely small biodegradable super-paramagnetic beads that do not affect cell function or viability. After magnetic labelling, the cells are passed through a separation column which serves to create a high-gradient magnetic field. The magnetically labelled cells are retained in the column, while non-labelled cells pass through. After removal of the column from the magnetic field, the magnetically retained cells can be eluted. There are two general approaches to magnetic cell sorting: depletion (negative selection) and positive selection. $CD4^+$ and $CD8^+$ T cells were isolated using positive MACS separation according to protocol provided by manufacturer and the magnetically retained cells were eluted and subjected to 40Gy irradiation to form a population of irradiated autologous APCs (used as a source of multiple costimulatory signals). When separated $CD8^+$ subsets were required, MACS separated $CD8^+$ T cells were labelled with anti-CD27 and

either anti-CD28 or anti-CD45RA and were fractioned on a FACSAria high speed cell sorter (BD Biosciences, Oxford, UK)

2.2 Flow Cytometry

2.2.1 Flow cytometry

Flow cytometry is a technique that allows for the rapid measurement of individual cells as they flow in a fluid stream one by one through a laser beam. Cells are analysed on the basis of their size and granularity and the use of antibodies conjugated to different fluorochromes enabled the identification of specific cell subsets and analysis of inhibitory receptor expression.

Four parameter flow cytometry was performed on a FACS Calibur flow cytometer (BD Biosciences, Oxford, UK). This machine has two lasers (488nm and 600-650nm) enabling the simultaneous measurement of 4 different coloured fluorochromes (channels FL1 – FL4). Five colour flow cytometry was conducted using a FACS LSR (BD Biosciences, Oxford, UK), which is equipped with 4 lasers (488nm, 325nm, 633nm and 594nm) enabling the concurrent quantification of 6 different fluorochromes.

2.2.2 Surface staining by direct immuno-fluorescence

For cell surface staining, cells were labelled at a density of 0.5-5 x 10^6 cells per tube in FACS tubes (BD Biosciences, Oxford, UK) at room temperature of 15 minutes. The expression of cell surface markers was determined using monoclonal antibodies directly conjugated to a specific fluorochrome. Utilising a combination of different fluorochromes FITC, PE, PerCP, APC and PE-Cy7, allowed for the detection of up to 5 different antigens simultaneously. After labelling, cell suspensions were washed in 5ml FACS test tubes (Falcon, BD Biosciences, Oxford, UK) with excess PBSA (1% w/v bovine serum albumin and 0.02% sodium azide, (both Sigma-Aldrich, Dorset, UK) in phosphate buffered saline (PBS) by centrifugation at 650×g for 5 minutes. The supernatant was carefully decanted and the cells resuspended in the residual fluid by vortexing and optimal concentrations of fluorochrome conjugated antibodies added. The tubes were vortexed and incubated in the dark at room temperature for 15 minutes. The cells were again washed in excess PBSA and the supernatant was decanted and the cells were then fixed, as detailed in section 2.2.5.

2.2.3 Intracellular staining

The detection of intracellular proteins, including cytokines, requires cells to be fixed and permeabilised to facilitate the access of antibody to intracellular structures whilst preserving the cells' morphological scatter characteristics. A modified protocol of the Caltag Fix and Perm cell permeabilisation kit (Caltag Laboratories, Paisley, UK) was used. If a combination of cell surface and intracellular antigen staining was required, cells were first incubated with cell surface antibodies as per the protocol above, washed with PBSA and centrifuged at 650 g for 5 minutes. After decanting the

supernatant, the cell pellet resuspended by the addition of 100µl reagent A (a fixation medium containing formaldehyde) whilst simultaneously vortexing and left to incubate in the dark at room temperature for 15 minutes. The cells were washed with excess PBSA and centrifuged at 650×g for 5 minutes. The supernatant was decanted and the cell pellet was resuspended with the addition of 100µl of reagent B (Permeabilisation medium) together with optimal concentrations of directly conjugated antibodies to intracellular antigens whilst simultaneously vortexing and left to incubate in the dark for 15 minutes at room temperature. The cells were then washed and fixed (as described in section 2.2.5).

2.2.4 Intranuclear staining

Ki67 antigen is the prototypic cell cycle related nuclear protein, expressed during all active phases of the cell cycle (G1, S, G2 and M phase) but is absent from resting (G0) cells. Ki67 is thus considered an excellent marker to determine the growth fraction of a given cell population (Scholzen and Gerdes, 2000). Ki67 staining was achieved by performing a modified protocol using the intranuclear Miltenyi FoxP3 Buffer Staining kit (Miltenyi Biotec, Surrey, UK). Cells were surface stained, as described in section 2.2.2, and washed by adding 2ml cold PBS and 650×g centrifugation for 5 minutes at 4°C. The supernatant was decanted and cells were resuspended in 0.5 ml cold freshly prepared Fixation/Permeabilisation solution and incubated on ice for 30 minutes. Cells washed in 0.5 ml cold permeabilisation buffer and centrifuged at 650×g for 5 minutes at 4°C. Supernatants were then decanted and 18µl anti-Ki67 antibody added before incubating on ice for 30 minutes. Cells were then washed in 0.5ml cold

permeabilisation buffer by centrifuging at 650×g for 5 minutes. Supernatants were then decanted and cells fixed, as described in section 2.2.5.

2.2.5 Flow Cytometric Analysis

After staining all samples were fixed with 100µl of 2% Paraformaldehyde (Sigma-Aldrich, Dorset, UK) in PBS and stored for at least 1 hour in the dark at 4°C prior to analysis. Acquisition and analysis of all data was undertaken using CellQuest software (BD Biosciences, Oxford, UK). Analysis was performed by gating on the live lymphocyte population based on the forward and side scatter profile that represents size and granularity, respectively to exclude dead cells and debris. Single colour fluorochrome controls were used prior to the start of sample acquisition in order to compensate between different channels to correct for overlapping emission spectra that can occur between different fluorochromes. Negative and isotype controls were used to delineate positive populations where positive and negative populations were not clearly distinguishable. The optimum concentration of each antibody was determined by preliminary titration. This was defined as the concentration that allowed the clearest distinction to be made between cells that stained positively and negatively for the marker under investigation. The antibodies used in flow cytometry are shown in Table 2.1

2.2.6 Determination of donor CMV status

10^6 PBMCs resuspended in RPMI containing 10% heat inactivated human AB serum were added to sterile FACS polypropylene tubes (Kendall, Tyco Healthcare group, Massachusetts, USA). The cells were stimulated with CMV-infected cell lysate (used at 1/10 dilution), which was prepared by infecting human embryonic lung fibroblasts with the Towne strain of CMV (European Collection of Animal Cell Cultures) at a multiplicity of infection of 2. After 5 days, the infected cells were lysed by repeated freeze-thaw cycles. PBMC were left unstimulated or stimulated with CMV lysate for 15 hrs at 37°C in a humidified CO_2 atmosphere, with 5 µg/ml Brefeldin A (Sigma-Aldrich, Dorset, UK) added after 2 hrs. Brefeldin A is a fungal metabolite that enhances the staining of intracellular cytokines by interfering with the vesicular transport of cytokines from the rough ER to the golgi complex, thereby preventing their secretion in antigen activated T cells. The cells were surface stained with anti-CD4, and then intracellularly stained with anti-IFNγ, as described in section 2.2.3. Uninfected cell lysates did not induce any IFNγ secretion. There was concordance between seropositivity and a positive IFNγ response in the blood for CMV (Fletcher et al., 2005). Furthermore, as a positive control, the superantigen Staphylococcal Exotoxin B (SEB; Sigma-Aldrich, Dorset, UK) was added to PBMCs at a final concentration of 1µg/ml and unstimulated cells were used as a negative control, as a guide for delineating positive and negative populations.

2.3 Flow Cytometric Analysis of Inhibitory Receptor Expression

2.3.1 Anti-CD3 Stimulating cells

100µl OKT3 diluted in PBS to 0.5µg/ml was added to each required well of a flat bottomed 96 well plate. PBS was added to unstimulated wells as a control. The plate was then incubated at 37°C for 2 hours before being washed 3 times with PBS. Required cells were then added to the relevant wells.

2.3.2 Analysis of Inhibitory Receptor Expression

PBMCs were stained directly *ex vivo* to examine KLRG1 expression and were stimulated with plate coated anti-CD3 (OKT3) at 37°C to enable CTLA-4 and PD-1 expression for 24hrs and 48hrs, respectively. PD-1 and KLRG1 expression was analysed using cell surface staining techniques as described in section 2.2.2, whereas CTLA-4 was determined intracellularly as detailed in section 2.2.3.

2.3.3 Determination of cytokine effects on inhibitory receptor expression

PBMC were stimulated in duplicate with plate coated anti-CD3 for 24 hours, as detailed in section 2.3.1, in the presence of cytokines (IL-6 10ng/ml, IL-7 10ng/ml, IL-10 50ng/ml, IL-15 10ng/ml, IL-17 1µg/ml, IL-21 0.2µg/ml, IFNα 500U/ml, TNFα 10ng/ml; all from PeproTech EC, London, UK) prior to inhibitory receptor staining as detailed in section 2.3.2

2.3.4 Effects of cytokine receptor blockade on inhibitory receptor expression

PBMC samples were stimulated in duplicate wells in 96-well flat bottomed plates which were anti-CD3 coated for 24 hours in the presence of 1µg/ml IFNαR block (Calbiochem, Nottingham, UK) or 50µg/ml IL-15R (R&D Systems, Oxford, UK block prior to inhibitory receptor staining as described in section 2.3.2.

2.4 Inhibitory receptor blockade

2.4.1 Inhibitory receptor blocking antibodies

Samples were blocked using 10µg/ml of each of E-cadherin (67A4, Chemicon International, Hampshire, UK), CTLA-4 (BN13, (BD Biosciences, Oxford, UK), PD-L1 (29F.2A3, kind gift from G Freeman), PD-L2 (24F, kind gift from Dr. G. Freeman, Dana-Farber Cancer Institute, Boston, MA) or the relevant isotype controls: IgG1 (MOPC31c, Sigma-Aldrich, Dorset, UK), IgG2a (Mg2a-53, Abcam, Cambridge, UK) or IgG2b (MPC-11, Abcam, Cambridge, UK), respectively. All blocking antibodies were azide free.

2.4.2 ^{3}H-Thymidine Incorporation assay with inhibitory receptor blockade

100µl of MACS purified $CD8^+$ T-cells (5×10^5 cells per well) were stimulated in triplicate with plate coated anti-CD3 and irradiated autologous APCs (5×10^5 per well) in 96-well flat-bottomed plates (Nunc, Roskilde, Denmark). Cells were cultured for 3 days at 37°C in a humidified 5% CO2 atmosphere in the presence of either inhibitory receptor blockade or isotype controls, as described in section 2.4.1. During

the last 18hrs of culture 10µl of 0.0025MBq [^{3}H]-thymidine (Amersham Biosciences, Buckinghamshire, UK) was added to each well. Cells were harvested onto glass microfibre filter strips using a cell harvester (Cambridge Technology, Massachusetts, USA) and counts per minute (cpm) from incorporated [^{3}H]-thymidine were determined by liquid scintillation counting. Proliferation was expressed either as the mean [^{3}H]-thymidine incorporation (cpm) of triplicate wells ±SD (standard deviation) or a proliferation index was calculated. Proliferation index was determined by calculating the ratio of cpm in cells proliferating in response to anti-CD3 stimulation alone versus the cpm in cells proliferating in response to anti-CD3 in combination with the inhibitory receptor blockade or isotype control.

2.4.3 Detection of Virus Specific CD8$^+$ T cell

PBMCs were incubated for 15mins at 37°C with the following PE- and APC-labelled HLA-A2 and HLA-B7 restricted pentamers to identify CMV specific CD8$^+$ T cells: A2-CMV (NLVPVMTV) and B7-CMV (TPRVTGGGAM) (ProImmune, Oxford, UK). Samples were then stained with anti-CD8 and any other required staining antibodies as described in section 2.2.

2.4.4 Effects of inhibitory receptor blockade on the proliferative responses of CD8$^+$ NLV/TPR specific T cells

Freshly isolated PBMCs from TPR$^+$ or NLV$^+$ donors were resuspended in RPMI containing 10% heat inactivated human AB serum and subjected to 0.2µg/ml

TPRVTGGGA or NLVPMVATV peptide stimulation (ProImmune, Oxford, UK) for 3 days in the presence of inhibitory receptor blockade or relevant isotype control before Ki67 staining, as described in section 2.2.4 on NLV/TPR$^+$ cells to determine virus specific proliferation.

2.4.5 Effects of inhibitory receptor block on the proliferation of CMV-pp65 specific CD8$^+$ T cells

Freshly isolated PBMCs from CMV$^+$ donors were resuspended in RPMI containing 10% heat inactivated human AB serum and subjected to 3 day 0.2µg/ml pp65 CMV peptide pool (JPT peptide technologies, Berlin, Germany) stimulus in the presence of inhibitory receptor blockade or relevant isotype control before Ki67 staining, as described in section 2.2.4, on CD8$^+$ T cells to determine virus specific proliferation.

2.4.6 Effects of inhibitory receptor blockade on the proliferative response of CMV specific cells from purified CD8$^+$ T cell subsets

MACS purified CD8$^+$ T cells from fresh blood were separated into its CD27/CD45RA subsets as described in section 2.1.7. 200,000 of these separated CD8$^+$ T cells were mixed with autologous irradiated APCs in a 1:1 ratio in RPMI supplemented with heat inactivated 10% human AB serum. These cells were stimulated in 96U round bottomed wells with 0.2µg/ml NLV or TPR peptide for 3 days, before the size and Ki67 expression of pentamer positive cells was determined.

2.4.7 Effects of inhibitory receptor blockade on CD4$^+$ CMV specific cell T proliferation

Freshly isolated PBMCs were resuspended in RPMI supplemented with 10% heat inactivated human AB serum. The cells were stimulated for 3 days with or without CMV lysate (used at a 1 in 10 dilution) in the presence of inhibitory receptor blocking antibody or the relevant isotype control. On day 2 the cells were restimulated with CMV lysate overnight in the presence of Brefeldin A. Cells were then surface stained with anti-CD4 and intranuclearly stained with anti-IFNγ and anti-Ki67 as described in section 2.2.4. CD4$^+$IFNγ$^+$ cells were identified as CMV specific and their expression of Ki67 represented the proliferative response of these cells.

2.5 Telomere and Telomerase

2.5.1 Measurement of telomere lengths

Telomere lengths measured *by* a 3 colour *flow cytometric detection of fluorescence in situ hybridization (flow-FISH.)* PBMCs were stained using anti-CD8-FITC and anti-CD4-biotin (Immunotech, Birmingham, UK) and streptavidin-Cy3 (Cedarlane Laboratories Ltd), then fixed and permeabilised (Fix and Perm Cell Permeabilisation Kit, Caltag Laboratories, Paisley, UK). Cells were washed once in hybridization buffer (70% formamide, 20 mM Tris, 150 mM NaCl, and 1% BSA) and then incubated at 82°C for 10 min with 0.75 μg/ml Cy5-conjugated telomeric (CCCTAA) peptide nucleic acid probe (Applied Biosystems, Warrington, UK). After rapid cooling on ice, the samples were hybridized for 1 hr at room temperature in the dark,

washed twice in post-hybridization buffer (70% formamide, 10 mMTris, 150 mM NaCl, 0.1% BSA, and 0.1% Tween 20) and then PBSA, and analysed immediately by flow cytometry. To ensure consistency of the results between experiments, two cryopreserved PBMC samples with known telomere fluorescence were used as standards. Results were obtained as median fluorescence intensity values, which could then be converted to telomere length in kilobases using a standard curve. The standard curve was constructed using 30 samples of varying telomere length analysed both by flow-FISH and telomeric restriction fragment analysis.

2.5.2 Measurement of Telomerase Activity

Purified $CD8^+$ T cell subset populations (2×10^5 cells), separated as described in section 2.1.7, were snap frozen after 3 day stimulation with either plate coated anti-CD3 or 0.2 µg/ml pp65 CMV peptide pool in the presence of autologous irradiated APCs and inhibitory receptor blocking antibodies. Using trypan blue exclusion counting (as described in section 2.1.3) and Ki67 staining the samples were adjusted to 500 $Ki67^+$ cells per reaction. The TeloTAGGG telomerase ELISA kit (Roche, Lewes, UK) was used to measure the telomerase activity according to the protocol provided by the manufacturer.

2.6 Statistical analysis

Statistical analysis was performed using GraphPad Prism version 4.05 for Windows (GraphPad Software, California, USA). For correlations between groups, the

D'Agostino and Pearson Omnibus Normality test was used to evaluate whether data sets were normally distributed. Statistical significance was evaluated using the Student's t test, if data followed a Gaussian distribution, and if data was also paired it was assessed using a Student's paired t test. Unpaired non-parametric data was evaluated using a Mann-Whitney U test, with a Wilcoxon matched paired test was used if data points also represented paired observations. Two tailed tests were used unless otherwise specified. For correlations, Pearson product-moment correlation coefficient was used to calculate r^2 and P values, and lines of best fit were generated using linear regression. Differences were considered significant when $P < 0.05$.

Antigen	Source	Clone	Isotype	Dilution
CD27-FITC	BD	M-T271	mIgG1	1/10
Ki67-FITC	BD	B56	mIgG1	1/10
CD8-FITC	BD	SK1	mIgG1	1/10
HLA-B7-FITC	AbD Serotec	BB7.1	mIgG1	1/10
CD27-PE	BD	M-T271	mIgG1	1/10
KLRG1-PE	H Pircher	13F12	mIgG1	1/10
PD-1-PE	G Freeman	EH12	mIgG2	1/10
CTLA-4-PE	BD	BN13	mIgG2a	1/10
HLA-A2-PE	AbD Serotec	BB7.2	mIgG1	1/10
CD8-PerCP	BD	SK1	mIgG1	1/10
CD4-PerCP	BD	SK3	mIgG1	1/10
CD27-APC	eBiosciences	O323	mIgG1	1/10

IFNγ-APC	BD	B27	mIgG2b	1/10
CD45RA-APC	Invitrogen	MEM-56	mIgG2b	1/10
CD4-PE-Cy7	BD	SK3	mIgG1	1/10
CD45RA-PE-Cy7	BD	L48	mIgG1	1/10
CD4-Biotin	Immunotech	13B8.2	mIgG1	

Table 2.01 List of antibodies used for flow cytometry

3 Characterisation of Inhibitory Receptor Expression Variations with Age & CMV Status

3.1 Introduction and Aims

An emerging field in age-associated immune decline is the role of immune inhibitory receptors. Augmented inhibitory receptor expression has been reported in aged mice and humans (as described in section 1.5.3) and following infection with various chronic pathogens, which may constitute a mechanism by which antigenic burden drives immune system ageing (as described in section 1.3.2). Moreover, cellular senescence has been demonstrated to be maintained by active signalling and be reversible in fibroblasts (d'Adda di et al., 2003). The critical signalling defect amongst highly differentiated $CD8^+$ T cells, that accumulate with ageing, is suggested as defective Akt activation (as described in section 1.2.3.2.3.2), which PD-1, CTLA-4 and KLRG1 can all mediate (as described in section 1.5.4). In summary, inhibitory receptor expression may increase with age and/or antigen load, which may define a reversible defect in T cell functionality of the aged.

In this chapter the role of the inhibitory receptors CTLA-4, PD-1 and KLRG1 in age-associated immune decline are initially assessed by characterising their expression with respect to age on $CD8^+$ and $CD4^+$ T cells. These studies are extended by further stratifying their expression based on donor CMV status and T cell differentiation stage. Finally, the functional relevance of these inhibitory receptor expression variations are elucidated using inhibitory receptor blocking antibodies.

3.2 Characterisation of functionally distinct T cell subpopulations at different differentiation stages

3.2.1 $CD8^+$ T cells

Forward scatter (FSC) versus side scatter (SSC) flow cytometry plots enable the evaluation of cells based upon size (FSC) and granularity (SSC). Lymphocytes form a tight population distinct from the larger monocytes, the smaller red blood cells and any highly granular apoptotic cells and cellular debris (Fig 3.01A). CD8 staining on gated live lymphocytes revealed 3 distinct populations: a $CD8^{hi}$ population representing $CD8^+$ T cells, a $CD8^{int}$ subset that defined NK cells and a $CD8^{lo}$ group that includes $CD4^+$ T cells and B cells (Fig 3.01B).

3.2.2 Utilisation of CD27/CD45RA markers to identify distinct T cell subsets

Both the $CD8^+$ and $CD4^+$ T cell pool are heterogeneous and include distinct subsets characterised by functional and phenotypic differences that can be defined on the basis of their heterogeneity in antigen expression. The phenotypic markers CD27 and CD45RA have been suggested as the most useful antigen combination to identify the four main T cell subsets: $CD27^+CD45RA^+$ (naïve, T_N), $CD27^+CD45RA^-$ (central memory, T_{CM}) $CD27^-CD45RA^-$ (effector memory, T_{EM}) and $CD27^-CD45RA^+$ (revertant memory, T_{REV}) (as described in section 1.2.3.1.7 and illustrated in Fig 3.01C,D).

3.2.3 CD27/CD28 markers distinguish T cells at distinct differentiation stages

For functional analyses, utilisation of the differentiation markers CD27 and CD28 were advantageous in that they allowed the enumeration of greater numbers of cells. $CD28^{+}CD27^{+}CD8^{+}$T cells were defined as early differentiated, with $CD27^{-}CD28^{+}$ as intermediate and $CD28^{-}CD27^{-}$ as late (described in section 1.2.3.1.7 and illustrated in Fig 3.01E,F). The rare $CD28^{+}CD27^{-}CD8^{+}$ T cell population is seldom found in sufficient numbers for isolation from most donors, though it has been characterised as having short telomeres, of comparable length with $CD28^{-}CD27^{-}CD8^{+}$ T cells, suggesting they too are a highly differentiated population (Plunkett et al., 2005).

CD8+

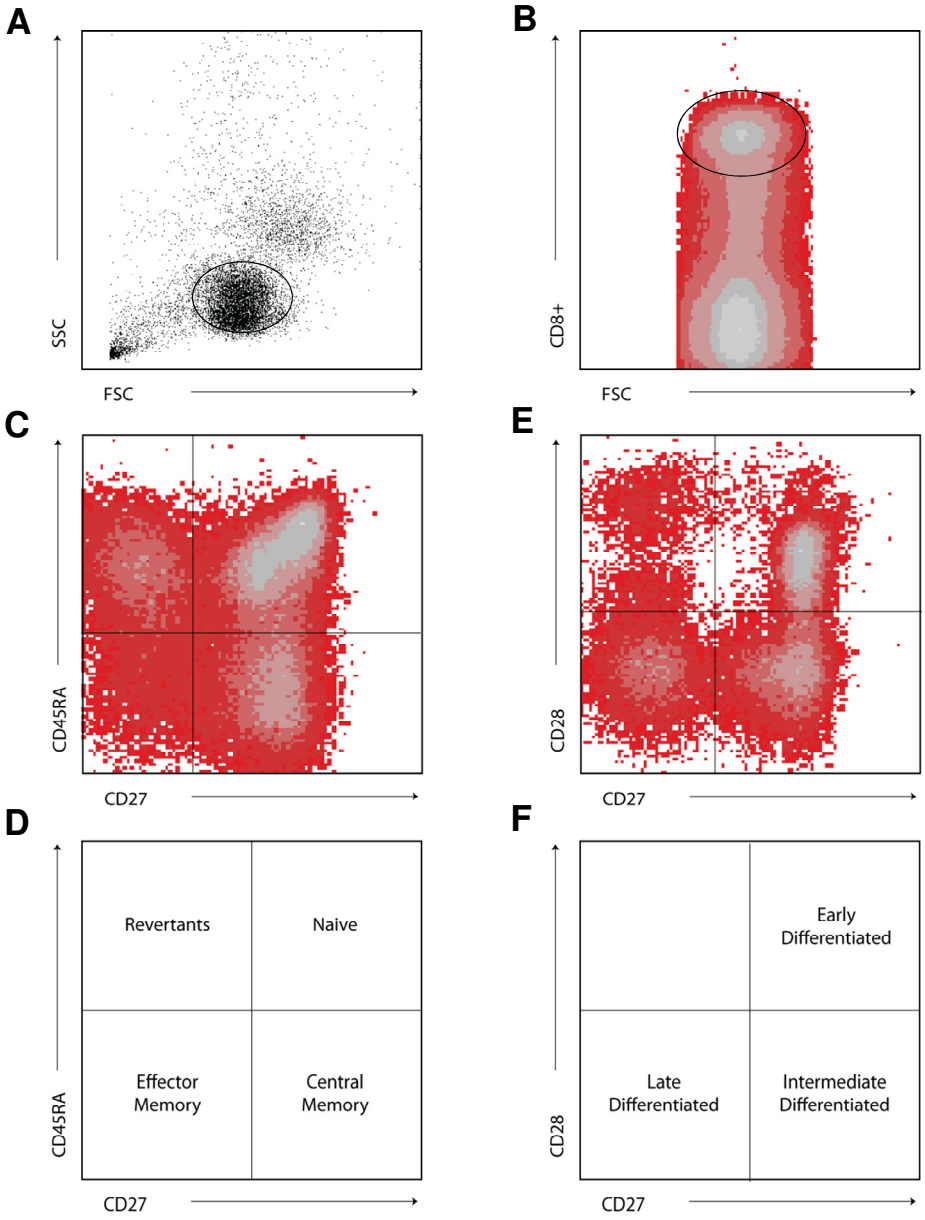


Figure 3.01. Identification of live lymphocytes and CD8+ T cell subpopulations by fluorescence cytometry

Purified PBMCs were stained with anti- CD8 PerCP, CD27 FITC and either CD45RA APC or CD28 APC. **(A)** Live lymphocytes were identified and gated on the basis of FSC/SSC profile in order to exclude cell debris. **(B)** A representative dot plot of the $CD8^+$ staining on live lymphocytes with $CD8^{intermediate}$ cells representing NK cells and $CD8^{hi}$ gated on and indicating $CD8^+$ T cells. **(C)** A scatter plot showing CD27/CD45RA staining gated on $CD8^+$ T cells. This enables the identification of different $CD8^+$ subpopulations including naive ($CD27^+CD45RA^+$), central memory ($CD27^+CD45RA^-$), effector memory ($CD27^-CD45RA^-$) and revertant memory cells ($CD27^-CD45RA^+$) as depicted in **(D)**. **(E)** An example of the CD27/CD28 profile of CD8+ T cells. The different populations identified are labelled early differentiated ($CD27^+CD28^+$), intermediate differentiated ($CD27^+CD28^-$), late differentiated ($CD27^-CD28^-$) and $CD27^-CD28^+$, as illustrated in **(F)**

3.2.4 CD4$^+$ T cells

CD4 staining on gated live lymphocytes reveal a CD4$^-$ population and a CD4$^+$ population that represents CD4$^+$ T cells (Fig 3.02A,B). CD27/CD45RA expression patterns define naïve, central memory, effector memory and CD45RA-revertant memory CD4$^+$ T cell populations in a manner analogous to CD8$^+$ T cells (Fierro et al., 2008) (Fig 3.02C,D). Additionally, the sequential loss of CD27 and CD28 can define different stages of CD4$^+$ T cell differentiation. However, in contrast to CD8$^+$ T cells, CD4$^+$ T cells lose CD27 expression before CD28, giving rise to the differentiation pathway CD27$^+$CD28$^+$→CD27$^-$CD28$^+$→CD27$^-$CD28$^-$ (as described in section 1.2.3.1.7 and illustrated in Fig 3.02E,F).

CD4+

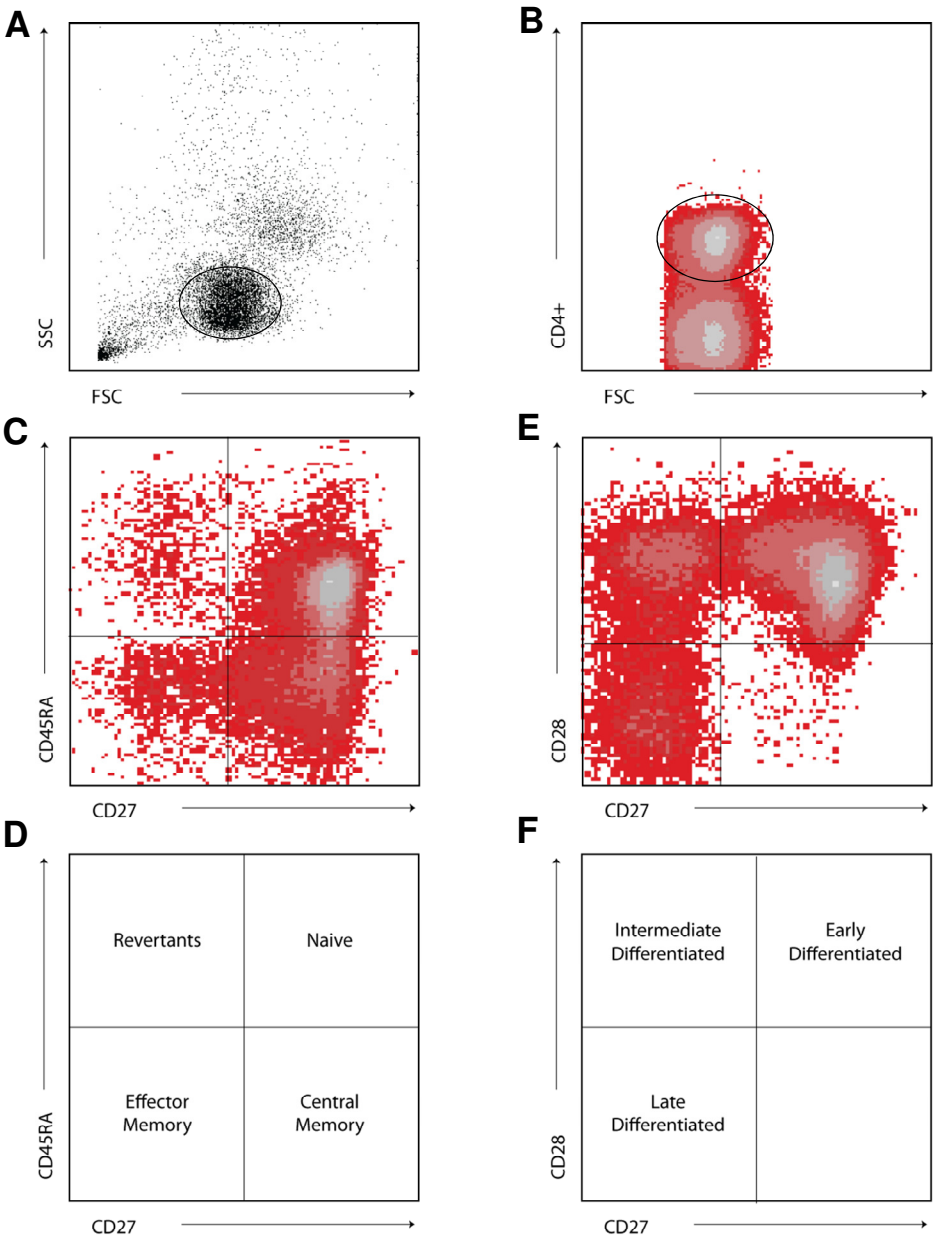


Figure 3.02. Using fluorescence cytometry for the identification of live CD4+ T cells at different differentiation stages as defined by CD27/CD45RA and CD27/CD28 phenotypic markers.

PBMCs were purified and stained with anti- CD4 PerCP, CD27 FITC and either CD45RA APC or CD28 APC. **(A)** A typical FSC/SSC profile upon which a live lymphocyte gate is drawn. **(B)** A representative FACS plot gated on live lymphocytes of the CD4 staining with $CD4^+$ gate depicted. **(C)** CD27/CD45RA staining on $CD4^+$ T cells enabling identification of naive ($CD27^+CD45RA^+$), central memory ($CD27^+CD45RA^-$), effector memory ($CD27^-CD45RA^-$) and revertant memory ($CD27^-CD45RA^+$) $CD4^+$ T cell subpopulations as depicted in **(D)**. **(E)** CD27 and CD28 staining gated on $CD4^+$ T cells permitting the distinguishment of early differentiated ($CD27^+CD28^+$), intermediate differentiated ($CD27^+CD28^-$), late differentiated ($CD27^-CD28^-$) and $CD27^-CD28^+$ $CD4^+$ T cells, as illustrated in **(F)**

3.3 $CD4^+$ T cells are more resistant to age and CMV associated phenotypic changes than $CD8^+$ T cells

3.3.1 Changes in $CD8^+$ T cell phenotype with age and CMV status, characterised using the phenotypic markers CD27/CD45RA

The percentage of naïve $CD8^+$ T cells diminished in a strong, highly significant manner with donor age and amongst CMV^+ donors compared with CMV^- in both young (<35yrs) and old (>65yrs) cohorts (Fig 3.03A representative example; Fig 3.03B cumulative data). However, the proportions of central and effector memory $CD8^+$ T cells do not significantly differ between these different cohorts (Fig 3.03C,D). Nevertheless, these reductions in naïve $CD8^+$ T cells are accompanied by significant increases in the percentages of CD45RA-revertant memory T cells with age (Fig 3.03E) and CMV status amongst young but not old donors (Fig 3.03E, $P < 0.05$ young, $P = 0.11$ old).

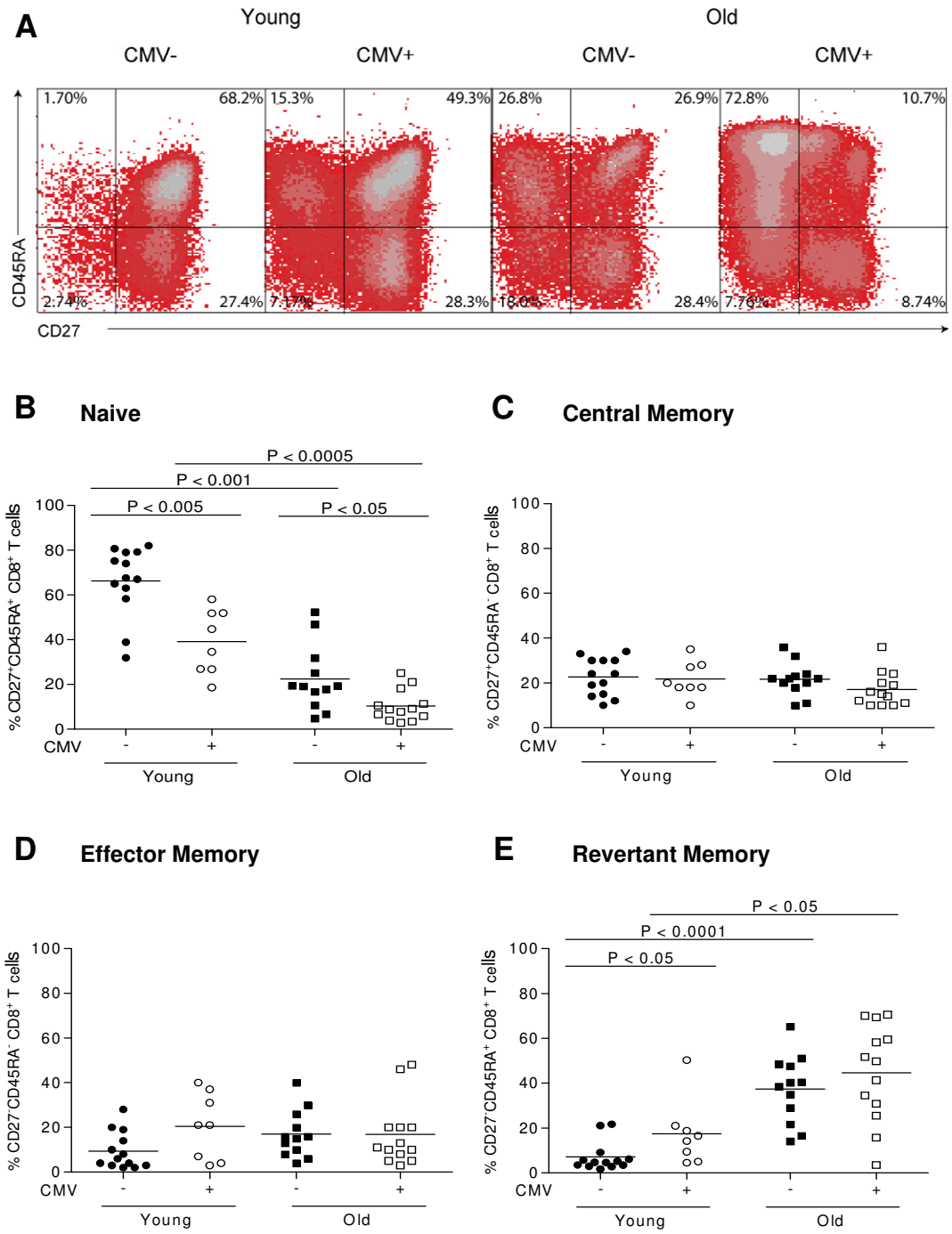


Figure 3.03. Change in phenotypic markers CD45RA and CD27 on CD8⁺ T cells with age and CMV.

Purified PBMCs were stained and analysed as described in Fig 3.01. **(A)** Representative examples of CD27/CD45RA staining on the on CD8⁺ T cells of a young CMV⁻ (n=13), young CMV⁺ (n=9), old CMV⁻ (n=10) and old CMV⁺ (n=20) donors. **(B)** Cumulative data showing the proportion of CD8⁺ T cells that are naive, **(C)** central memory, **(D)** effector memory and **(E)** revertant memory cells stratified on the basis of age and CMV status. Filled symbols indicate CMV negative individuals and open symbols CMV positive individuals. Young donors are <35 years and old donors >65. Horizontal lines illustrate mean values. The P values were calculated using a Mann-Whitney U test. Significant differences are shown.

3.3.2 Age and CMV associated alterations in the differentiation status of the CD8$^+$ T cell pool as defined by CD27/CD28 expression

Utilising the alternative differentiation markers CD27 and CD28, a similar age and CMV associated remodelling of the CD8$^+$ T cell compartment can be observed (Fig 3.04A, representative example). Indeed, the proportion of early differentiated CD8$^+$ T cells undergo a significant diminution with age and CMV status (Fig 3.04B), which is accompanied by a significant accumulation of late differentiated CD8$^+$ T cells among old donors and also among CMV$^+$ young compared with their CMV$^-$ counterparts (Fig 3.04D). The proportions of CD8$^+$ T cells belonging to the intermediately differentiated CD27$^-$CD28$^+$ subset and the rare CD27$^+$CD28$^-$ population did not significantly vary between the different cohorts (Fig 3.04 C,E).

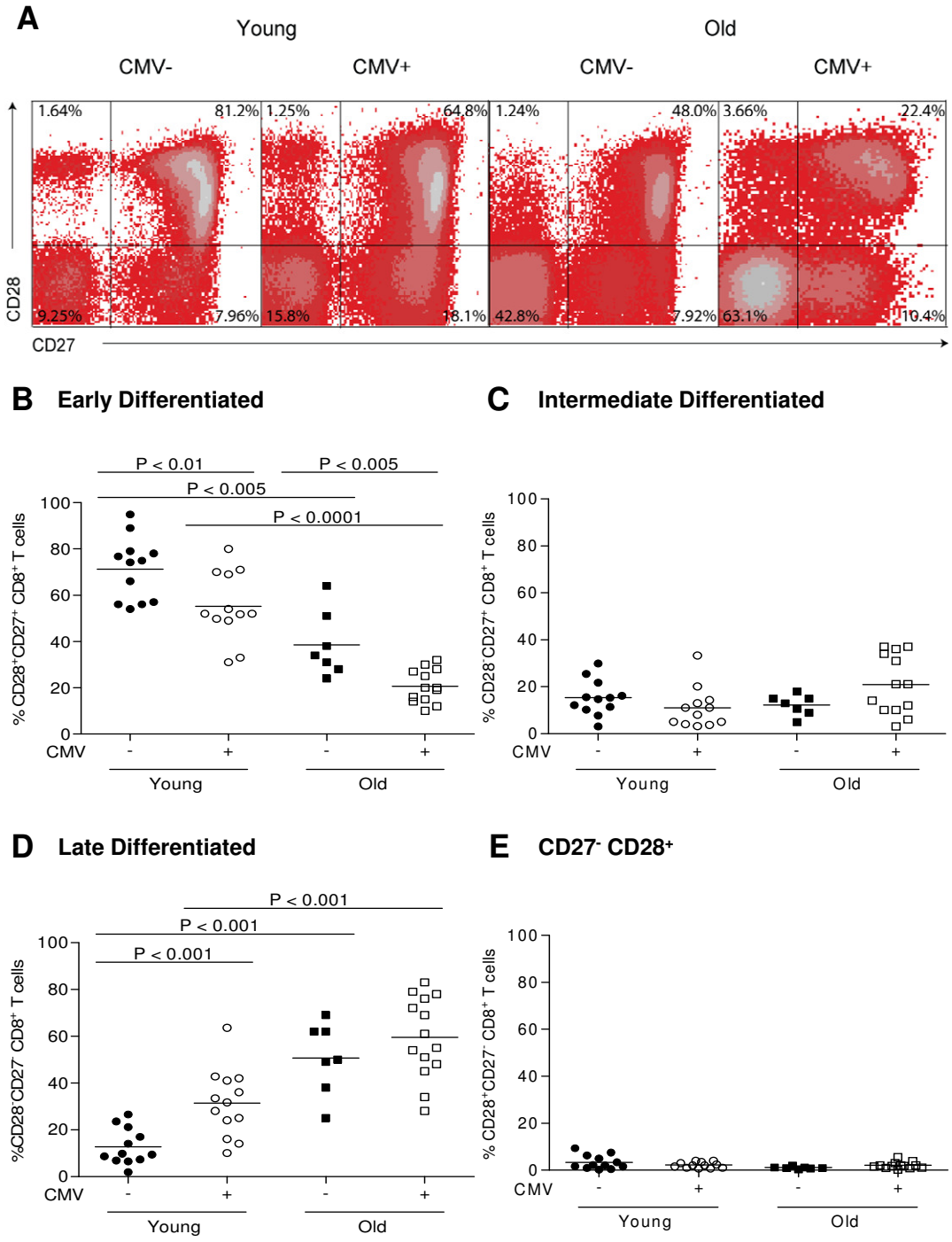


Figure 3.04. Alterations to CD8⁺ T cell phenotype, as defined by CD28 and CD27, stratified by donor age and CMV status.

CD8/CD27/CD28 PBMC staining performed in accordance with Fig 3.01. (A) Illustrative examples of the CD27/CD28 profile of CD8⁺ T cells from a young CMV⁻, (n=12) young CMV⁺ (n=13), old CMV⁻ (n=8) and old CMV⁺ (n=13) donors **(B)** Pooled data illustrating how the proportion of early, **(C)** intermediate, **(D)** late differentiated and **(E)** CD27⁻CD28⁺ CD8⁺ T cells changes with ageing and CMV status. Filled symbols indicate CMV negative individuals and open symbols CMV positive individuals. Young donors are <35 years and old donors >65. Horizontal lines indicate mean values. A Mann-Whitney U test was used to calculate P values and only significant differences are shown.

3.3.3 Phenotypic variation of CD4$^+$ T cells with donor age and CMV status, stratified using the markers CD27/CD45RA

Although reductions in naïve T cells that approached significance could be observed amongst the CD4$^+$ T cell compartment, both with age (Fig 3.05A, representative example; Fig 3.05B, P = 0.10 amongst CMV$^+$ donors, P = 0.30 within CMV$^-$ cohort) and CMV seropositivity (Fig 3.05B, P = 0.16 old, P = 0.07 young), these differences were much smaller both in magnitude and significance, when compared with those among CD8$^+$ T cells. Moreover, this was accompanied primarily by accumulation of central memory CD4$^+$ cells, in young CMV$^+$ (Fig 3.05C, P < 0.05 compared with young CMV$^-$) and old CMV$^-$ donors (Fig 3.05C, P = 0.07 compared with young CMV$^-$), rather than CD45RA-revertant memory cells, as observed amongst the CD8$^+$ T cell compartment.

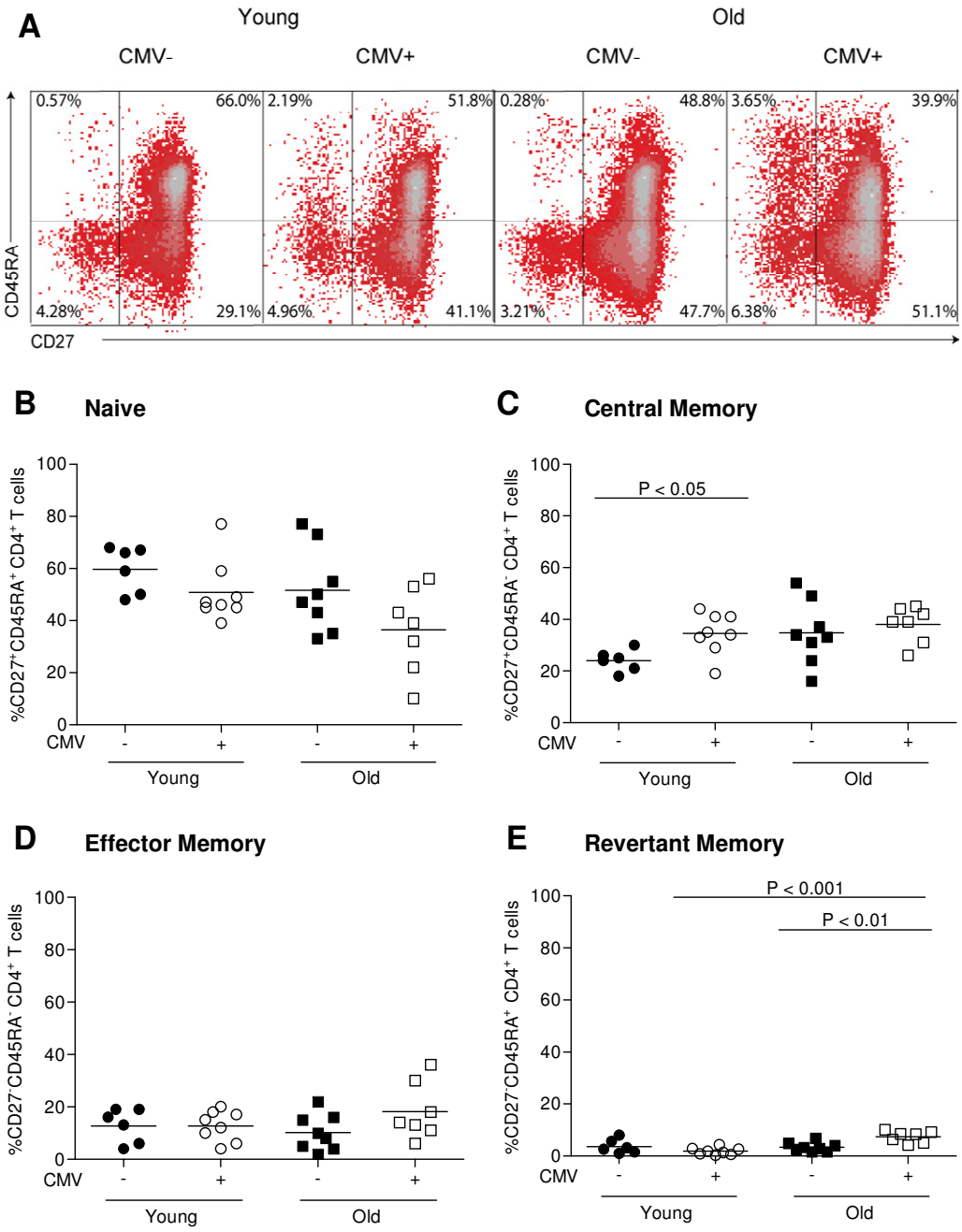


Figure 3.05. CMV and age related changes in differentiation status of CD4⁺ cells defined by their CD27/CD45RA expression .

PBMCs subjected to flow cytometric experiments as in Fig 3.02. **(A)** Dot plots showing expression of the phenotypic markers CD27 and CD45RA on a typical young CMV⁻, (n=6) young CMV⁺ (n=8), old CMV⁻ (n=8) and an old CMV⁺ (n=7) donors' CD4⁺ T cells. **(B)** Percentages of CD4⁺ T cells that are naive, **(C)** central memory, **(D)** effector memory and **(E)** revertants in young and old, CMV negative and positive donors. Filled symbols indicate CMV negative subjects and open symbols CMV positive individuals. Young donors are <35 years and old donors >65. Mean values are depicted by horizontal bars. Only significant differences are shown, calculated using a Mann-Whitney U test

3.3.4 Alterations in the differentiation state of CD4$^+$ T cells, as defined by CD27/CD28 expression, with increasing age and CMV status

Once again, in contrast to the dramatic reduction observed in early differentiated CD8$^+$ T cells, the CD28$^+$CD27$^+$ subset of the CD4$^+$ T cell compartment in CMV$^+$ and aged donors undergoes little variation, with significant reductions only being observed amongst CMV$^+$ old subjects (Fig 3.06A, representative example; Fig 3.06B, $P < 0.05$ compared with CMV$^-$ old, $P = 0.07$ compared with CMV$^+$ young). The proportion of intermediate differentiated or CD27$^-$CD28$^+$ CD4$^+$ T cells did not vary with age or CMV status (Fig 3.06C,E) and late differentiated CD4$^+$ T cells were only observed in significant numbers in CMV$^+$ old donors (Fig 3.06D, $P < 0.05$ compared with CMV$^-$ young and $P = 0.07$ relative to CMV$^+$ young subjects).

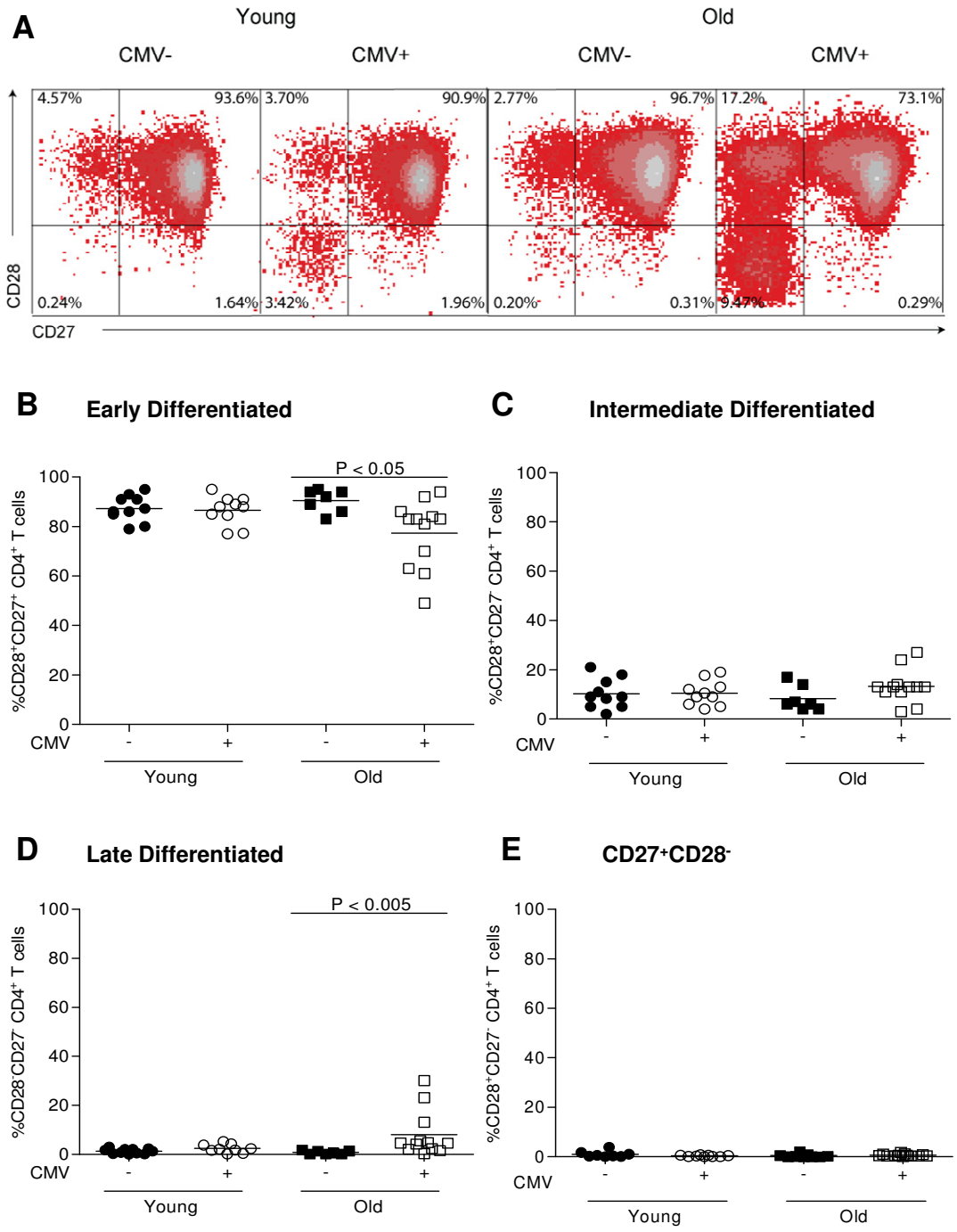


Figure 3.06. CMV and age related variations in CD4⁺ subset distribution according to CD28 and CD27 expression

PBMCs stained with anti- CD27, CD28 and CD4 before being analysed as stated in Fig 3.02. **(A)** Demonstrative dot plots of a young (<35) CMV⁻ (n=10) and CMV⁺ (n=10), and an old (>65) CMV⁻ (n=7) and CMV⁺ (n=12) subjects' CD27/CD28 profiles gated on CD4⁺ T cells **(B)** Pooled data showing how the proportion of early, **(C)** intermediate **(D)** late differentiated and **(E)** CD27⁺CD28⁻ CD4⁺ T cells changes with age and CMV status Filled symbols indicate CMV negative individuals and open symbols CMV positive individuals. Horizontal lines depict mean values. The P values were calculated using Mann-Whiney U test and only differences that are significant are shown.

3.4 Age and CMV Associated Upregulation of Different T cell Inhibitory Receptors on $CD4^+$ and $CD8^+$ T cells

3.4.1 CTLA-4 expression can be stratified on the basis of donor age only on $CD8^+$ T cells

The role of inhibitory receptors in age onset immune decline was initially assessed by characterising expression of these receptors on T cells with respect to age. The majority of CTLA-4 molecules are localised in intracellular stores and CTLA-4 is only transiently expressed at low levels on the cell surface following T cell activation (Linsley et al., 1996) and cannot be demonstrated in unstimulated PBMCs using flow cytometry (Steiner et al., 1999). Additionally, the kinetics of CTLA-4 intracellular T cell expression after stimulation parallels those of surface expression (Alegre et al., 1996; Leng et al., 2002b; Miller et al., 2002). Therefore, CTLA-4 expression was examined using intracellular staining techniques on PBMCs, stimulated with anti-CD3 for 24hrs to enable peak expression. CTLA-4 expression increased with age in a strong and significant manner on $CD8^+$ T cells, independent of CMV status (Fig 3.07A, representative example; Fig 3.07B pooled data). In contrast, no age-associated CTLA-4 expression variations were observed on the $CD4^+$ pool in either CMV^- or CMV^+ subjects (Fig 3.07C, representative example; Fig 3.07D cumulative data). Grouping donors based on their CMV status revealed that CTLA-4 expression on $CD8^+$ and $CD4^+$ T cells is significantly higher on CMV^+ individuals compared with their CMV^- counterparts (data not shown, $CD8^+$ $P< 0.0001$; $CD4^+$ $P < 0.005$).

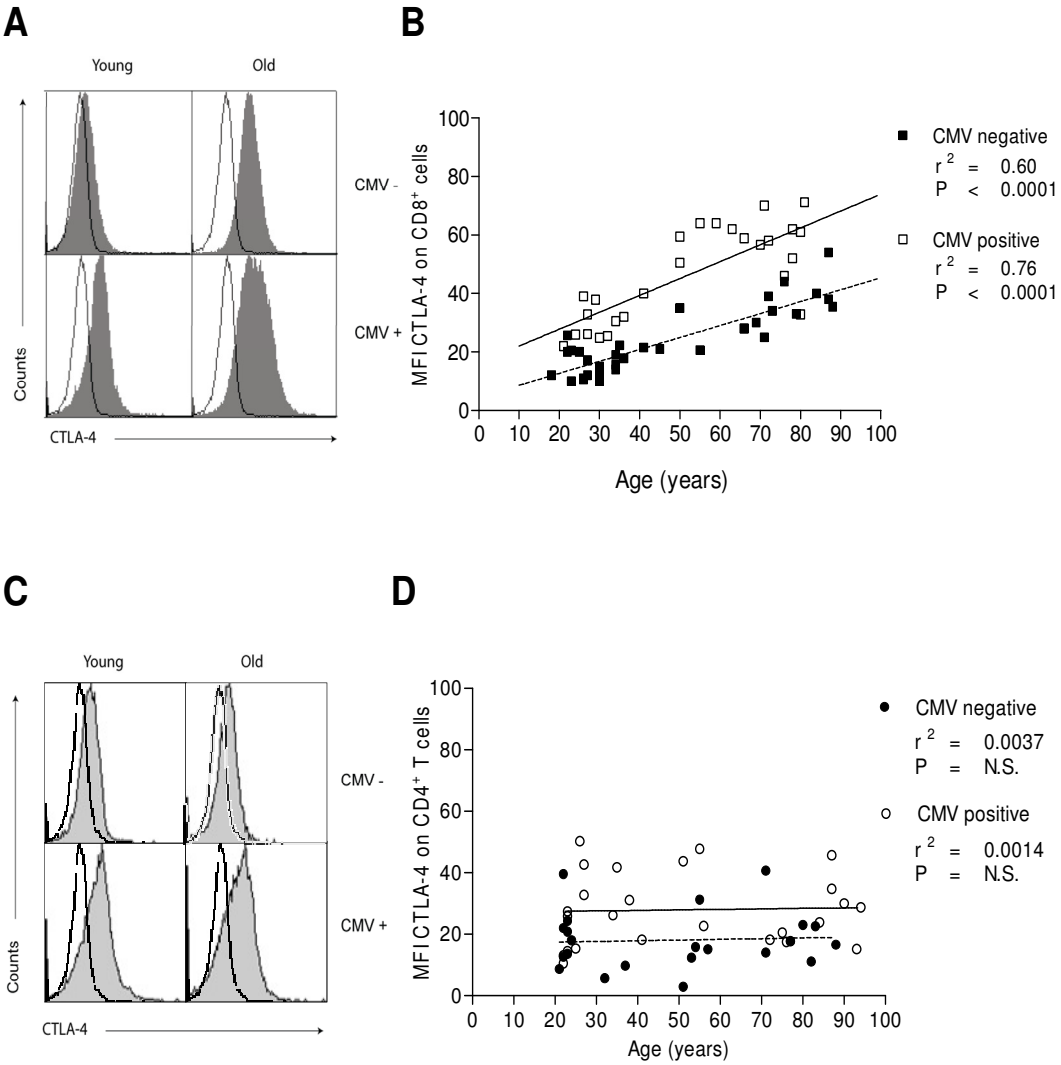


Figure 3.07. CTLA-4 expression is positively correlated with CMV status in CD8⁺ and CD4⁺ T cells but with age only in CD8⁺ T cells

CTLA-4 expression was measured by intracellular flow cytometry on PBMCs stimulated for 24hr with OKT3 and then stained with anti-CTLA-4 PE and either anti-CD4 or CD8 PerCP. **(A)** Representative examples of CTLA-4 staining in CMV^- and CMV^+ young and old donors' $CD8^+$ and **(B)** $CD4^+$ T cells. Filled plots indicate CTLA-4 and unfilled plots represent isotype control staining **(C)** Cumulative data, correlated by age and CMV status, depicting CTLA-4 expression on $CD4^+$ and **(D)** CD8 T cells. Filled symbols indicate CMV negative individuals and open symbols CMV positive individuals. Line of best fit generated using linear regression. P and r^2 values calculated using Pearson's correlation.

3.4.2 PD-1 expression displays no age-associated variation on either CD4$^+$ or CD8$^+$ T cells

Similar to CTLA-4, PD-1 is poorly expressed on resting lymphocytes (Parry et al., 2005) but, in contrast to CTLA-4, it undergoes a strong and sustained surface upregulation after cellular activation. Therefore, PD-1 expression was examined using surface staining techniques following 48hrs anti-CD3 stimulation of PBMCs to enable maximal expression. Unlike CTLA-4, PD-1 expression on CD8$^+$ T cells is not age dependent (Fig 3.08A, representative example; Fig 3.08B, grouped data). Similarly no age dependent variation in PD-1 expression is observed amongst CD4$^+$ T cells (Fig 3.08C, representative example; Fig 3.08D cumulative data). However, CMV infection upregulates PD-1 expression in both CD8$^+$ and CD4$^+$ T cells (data not shown, CD8$^+$ $P < 0.0001$; CD4$^+$ $P < 0.0005$).

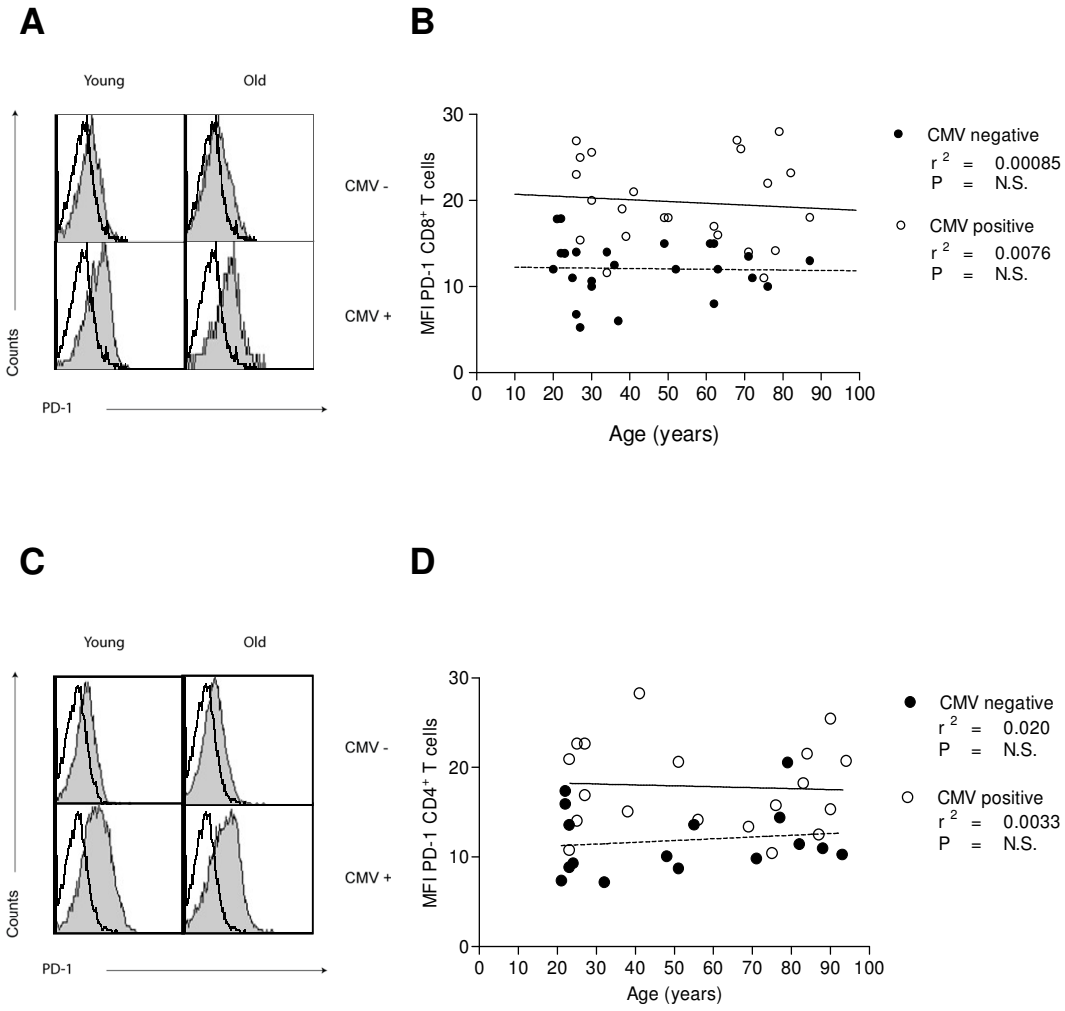


Figure 3.08. PD-1 expression on CD8⁺ and CD4⁺ T cells is significantly elevated in CMV⁺ individuals but is independent of age

PBMCs were stimulated for 48hrs with anti-CD3 and then surface stained with anti-PD-1 PE and either anti-CD8 or CD4 PerCP. **(A)** Representative histograms of PD-1 staining on $CD8^+$ and **(B)** $CD4^+$ T cells from a young and old, CMV^- and CMV^+ donors with the filled bars depicting PD-1 expression and the isotype control staining being represented by an unfilled plot. **(C)** Pooled data, demonstrating how PD-1 expression changes with age and CMV status on $CD8^+$ and **(D)** $CD4^+$ T cells. Filled symbols indicate CMV negative individuals and open symbols, CMV positive individuals. Linear regression used to generate line of best fit and Pearson's correlation used to calculate P and r^2 values

3.4.3 KLRG1 expression increases with age more dramatically on CD8$^+$ than CD4$^+$ T cells

KLRG1 is constitutively expressed on the T cell surface and its expression was examined directly *ex vivo*, without stimulus. Unlike the expression patterns of CTLA-4, and PD-1, clearly identifiable KLRG1$^+$ and KLRG1$^-$ populations could be observed and thus the percentage of cells that express KLRG1 was measured. As observed for CTLA-4, KLRG1 expression on CD8$^+$ T cells significantly increased with age (Fig 3.09A, representative example; Fig 3.09B, pooled data). However, the age-associated increases in KLRG1 expression on CD4$^+$ T cells were much more modest and only reached significance amongst CMV$^+$ donors (Fig 3.09C, representative example; Fig 3.09D, grouped data). KLRG1 is also expressed at augmented levels on the CD8$^+$ and CD4$^+$ T cells of CMV$^+$ compared with CMV$^-$ donors (data not shown, CD8$^+$ $P < 0.0001$; CD4$^+$ $P < 0.0005$).

KLRG1

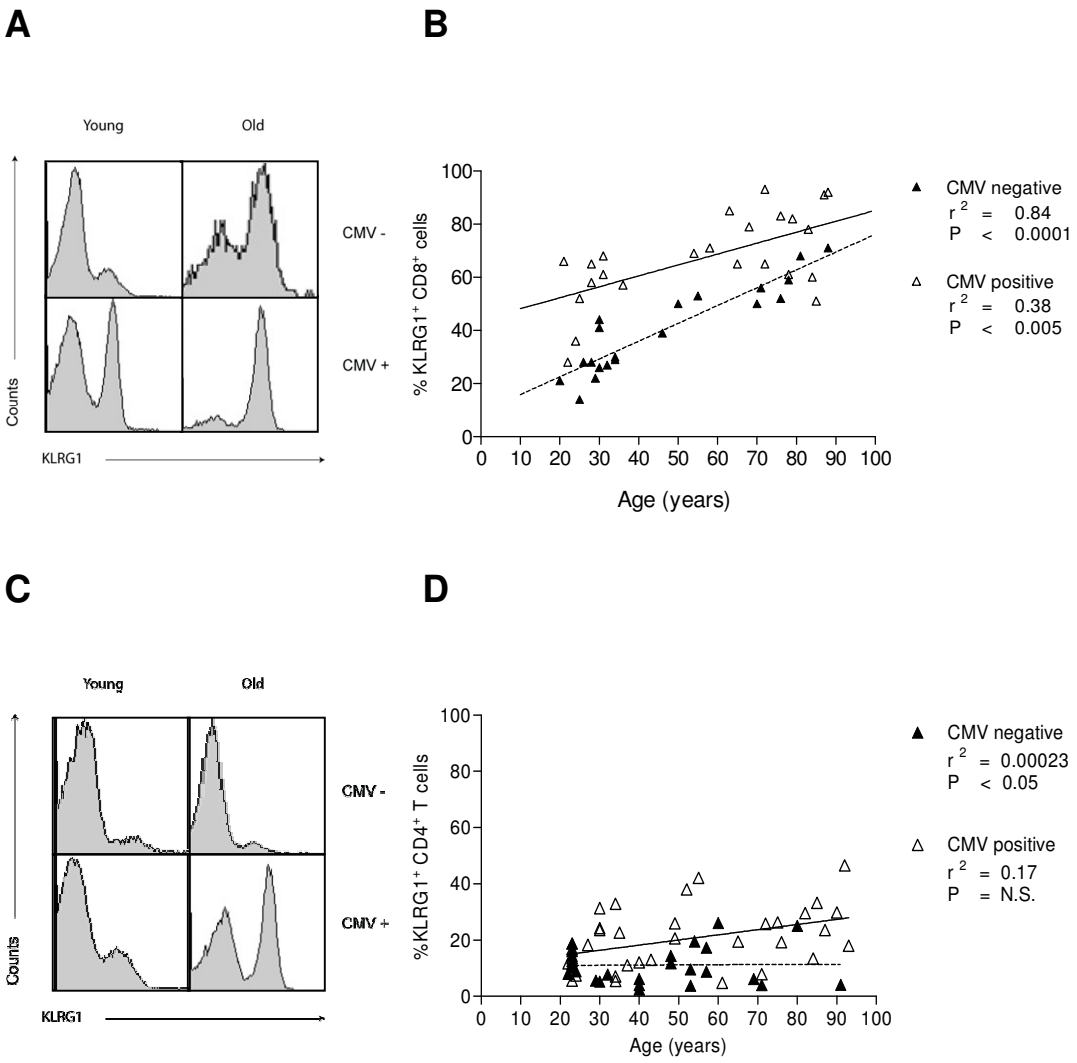


Figure 3.09. Expression of KLRG1 on CD4⁺ and CD8⁺ T cells with age and CMV status.

KLRG1 expression was determined using surface flow cytometry on unstimulated PBMCs following staining with anti-KLRG1-PE and either CD8 or CD4 PerCP. **(A)** FACS plots of KLRG1 expression gated on $CD8^+$ or **(B)** $CD4^+$ T cells from representative young, old, CMV^- and CMV^+ donors. Unfilled plots represent isotype control and filled plots indicate KLRG1 staining. **(C)** Pooled data illustrating KLRG1 expression on $CD8^+$ and **(D)** $CD4^+$ T cells, stratified by age and CMV status. Filled symbols indicate CMV negative and open symbols CMV positive individuals. Line of best fit generated using linear regression. P and r^2 values calculated using Pearson's correlation.

3.5 Defining Inhibitory Receptor Expression on Different T cell Subsets According to Donor Age

3.5.1 Determination of inhibitory receptor expression on distinct T cell subsets

To better characterise the nature of these age- and CMV-associated expression changes, inhibitory receptor expression was further characterised upon different T cell phenotypic and differentiation stages, amongst both young and old individuals. In the case of CTLA-4 and KLRG1, this was achieved by staining cells with CD27/CD45RA or CD27/CD28 markers, following 24 hour stimulation (in the case of CTLA-4) or directly *ex vivo* (for KLRG1), as described in Figs 3.01 and 3.02. However, in order to measure PD-1 expression, PBMCs were stimulated for 48 hours, which was sufficient time to induce T cell differentiation resulting in dramatic changes in T cell phenotype (Fig 3.10A). Therefore to measure PD-1 expression at different $CD8^+$ T cell differentiation stages, purified $CD8^+$ T cell subsets were stimulated with autologous irradiated APCs, as a source of multiple costimulatory molecules, for 48 hours before staining. However, this method is significantly more labour intensive, therefore when performing the analysis on $CD4^+$ T cells, it was found that stimulating PBMCs with anti-CD3 for 24 hours (Fig 3.10B) could generate a comparable pattern of PD-1 expression to that generated using FACS sorted $CD4^+$ T cell subsets stimulated for 48 hours (Fig 3.10C). Therefore this method was used to determine PD-1 expression at different $CD4^+$ T cell phenotypic stages.

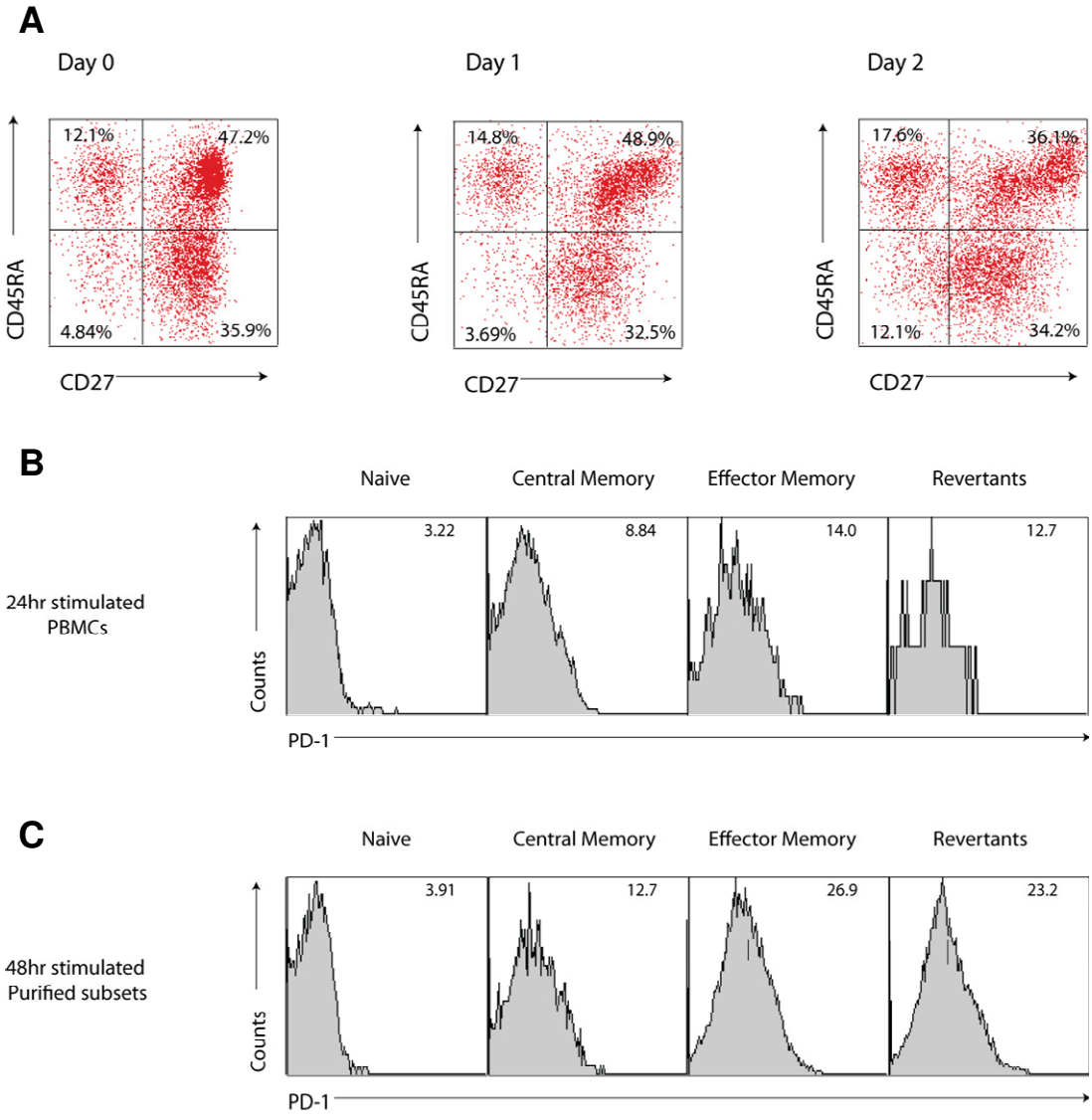


Figure 3.10. Similar PD-1 expression profiles generated on CD4⁺ T cell subsets after stimulating PBMCs or FACS Aria purified subsets with anti-CD3.

(A) Purified PBMCs stimulated with anti-CD3 and stained with anti-CD4 PerCP, CD45RA APC and CD27 FITC. CD27/CD45RA plots gated on CD4⁺ T cells are displayed **(left panel)** 0 hours, **(middle panel)** 24 hours and **(right panel**) 48 hours after stimulation. **(B)** PBMCs were either stimulated with OKT3 for 24hrs or **(C)** Purified **C**D4⁺ subsets, defined using CD27 and CD45RA, were stimulated with OKT3 and autologous irradiated APCs for 48hrs. The cells were then stained with anti-CD27 FITC, CD4 PerCP, CD45RA APC and analysed by flow cytometry. The mean fluorescent intensity of PD-1 expression on each subset is indicated.

3.5.2 CTLA-4, PD-1 and KLRG1 expression varies with CD8$^+$ T cell differentiation states on young and old individuals

CTLA-4 expression on CD8$^+$ T cells is upregulated on each differentiation stage as defined by CD27/CD45RA, except CD45RA-revertant memory cells, in old subjects compared to young (Fig 3.11A) with expression concentrated primarily on central and effector memory subsets among young individuals, whereas in old individuals naïve and central memory subsets express the highest levels. The pattern of PD-1 expression on different CD8$^+$ T cell differentiation stages does not significantly differ between old and young donors, with both age groups expressing peak PD-1 levels on central and effector memory T cells (Fig 3.11B). KLRG1 expression dramatically increases as CD8$^+$ T cells differentiate with no differences in expression between young and old donors (Fig 3.11C).

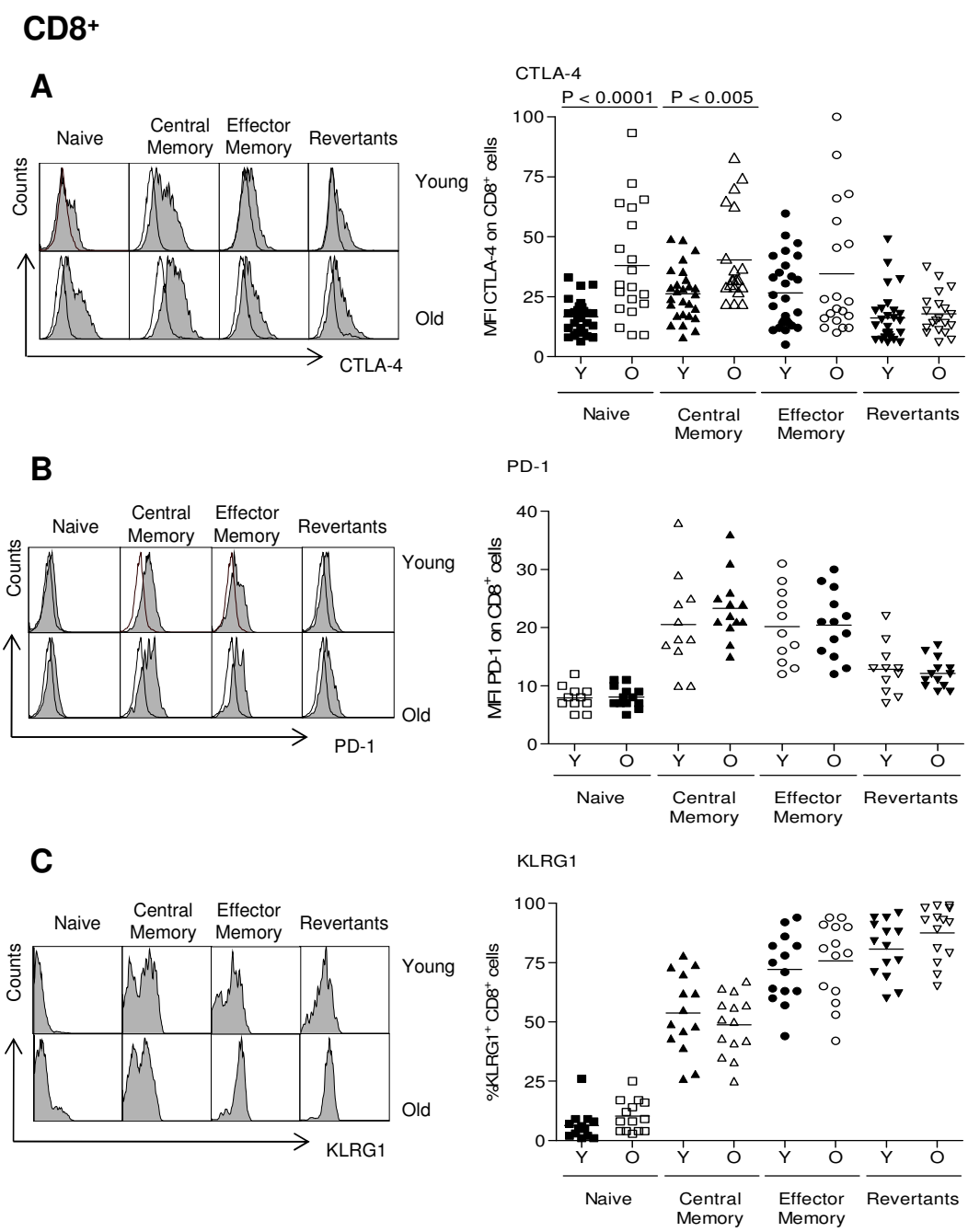


Figure 3.11 Differences in CTLA-4, PD-1 and KLRG1 expression on CD8+ T cells stratified by the phenotypic markers CD27 and CD45RA in old and young donors

CTLA-4, PD-1 and KLRG1 expression was quantified as described in Fig 3.07, 3.08 and 3.09, respectively, on CD8+ T cells at different phenotypic stages using the makers CD27 and CD45RA. **(A, left panel)** Histograms from a representative young and old donor illustrating CTLA-4, **(B, left panel)** PD-1 and **(C, left panel)** KLRG1 staining at different CD8+ T cell phenotypic stages based on CD27/CD45RA staining. **(A, right panel)** Pooled data displaying CTLA-4 **(B, right panel)** PD-1 and **(C, right panel)** KLRG1 expression of CD8+ T cells stratified by CD27 and CD45RA expression in young as compared with old donors. Filled symbols represent young donors, Y, and open symbols signify old individuals, O. Horizontal lines represent mean values. The P values were calculated using Student's t-test. Significant differences between young and old donors are shown.

3.5.3 Inhibitory receptor expression stratified by CD27/CD45RA expression on CD4$^+$ T cells on young and old donor cohorts

Unlike the results observed for CD8$^+$ T cells, CTLA-4 expression on CD4$^+$ T cell subsets does not significantly differ between young and old donors, but displays a similar pattern to that of CD8$^+$ T cell subsets of young individuals, with expression peaking in the central memory subset (Fig 3.12A). PD-1 expression on CD4$^+$ T cells forms a completely different pattern to that of CD8$^+$ cells with expression being greatest on T_{EM} and T_{REV} cells (Fig 3.12B). PD-1 expression was also augmented on the naïve CD4$^+$ cells of old individuals compared with their young counterparts (Fig 3.12B). KLRG1 expression on CD4$^+$ T cells increases with differentiation in a similar manner to CD8$^+$ T cells but the increases are much less pronounced (Fig 3.12C), with significantly elevated expression observed in effector memory and CD45RA-revertant memory subsets of old donors compared to young.

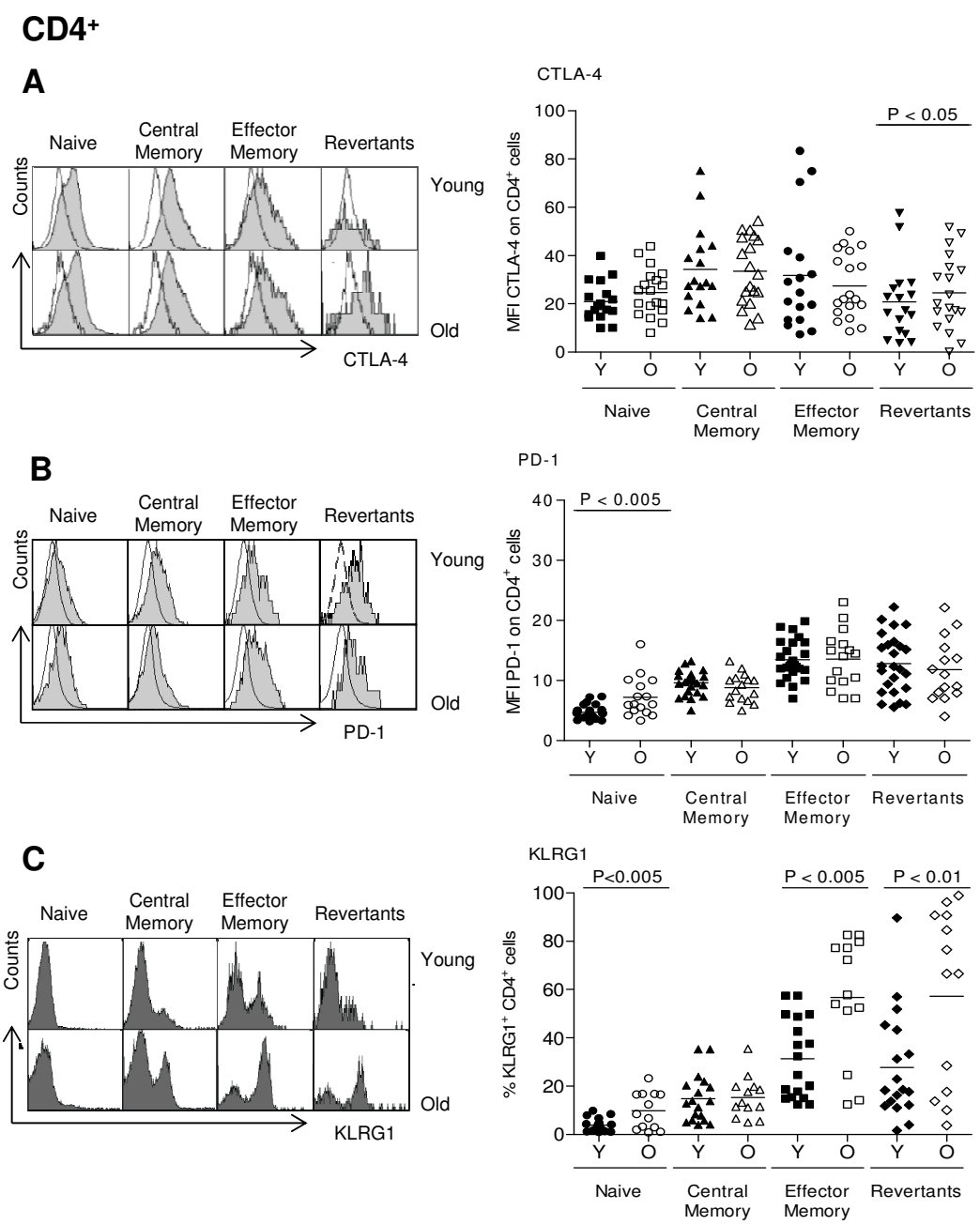


Figure 3.12. The expression of CTLA-4, PD-1 and KLRG1 on CD4+ T cells based on CD27 and CD45RA phenotypic markers amongst the old and young.

PBMCs from young (<35 years) and old (>65) were stimulated stained and analysed by flow cytometry for CTLA-4, PD-1 and KLRG1 expression defined by CD27/CD45RA, as described in the previous figure but using ani-CD4 PerCP rather than anti-CD8 PerCP. **(A, left panel)** Representative FACS plots of CTLA-4, **(B, left panel)** PD-1 and **(C, left panel)** KLRG1 expression in a young and an old donor on the basis of the phenotypic markers: CD27 and CD45RA. **(A, right panel)** Cumulative data is plotted as CTLA-4, **(B, right panel)** PD-1 and **(C, right panel)** KLRG1 expression on different CD4+ T cell subsets defined by CD27 and CD45RA expression un young and old individuals. Filled symbols represent young donors, Y, and open symbols signify old individuals, O. Horizontal lines depict mean values. P values were calculated using Student's t-test. Significant differences between young and old donors are shown.

3.6 Stratifying inhibitory receptor expression on different T cell subpopulations on the basis of donor age and CMV status

3.6.1 CTLA-4

Inhibitory receptor expression was characterised with respect to CMV status in young and old donors at different phenotypic stages. CMV positive individuals display similar CTLA-4 expression patterns, with respect to T cell differentiation, compared with their age matched CMV negative counterparts. Nevertheless, augmented CTLA-4 expression levels are observed amongst young CMV^+ versus CMV^- donors which reached significance among several subsets (Fig 3.13A,B left panels). Amongst old donors, the magnitude of CTLA-4 expression appears greater amongst CMV^+ donors, but this increase only reaches significance in $CD8^+$ T_{CM} cells (Fig 3.13A,B right panels).

CTLA-4

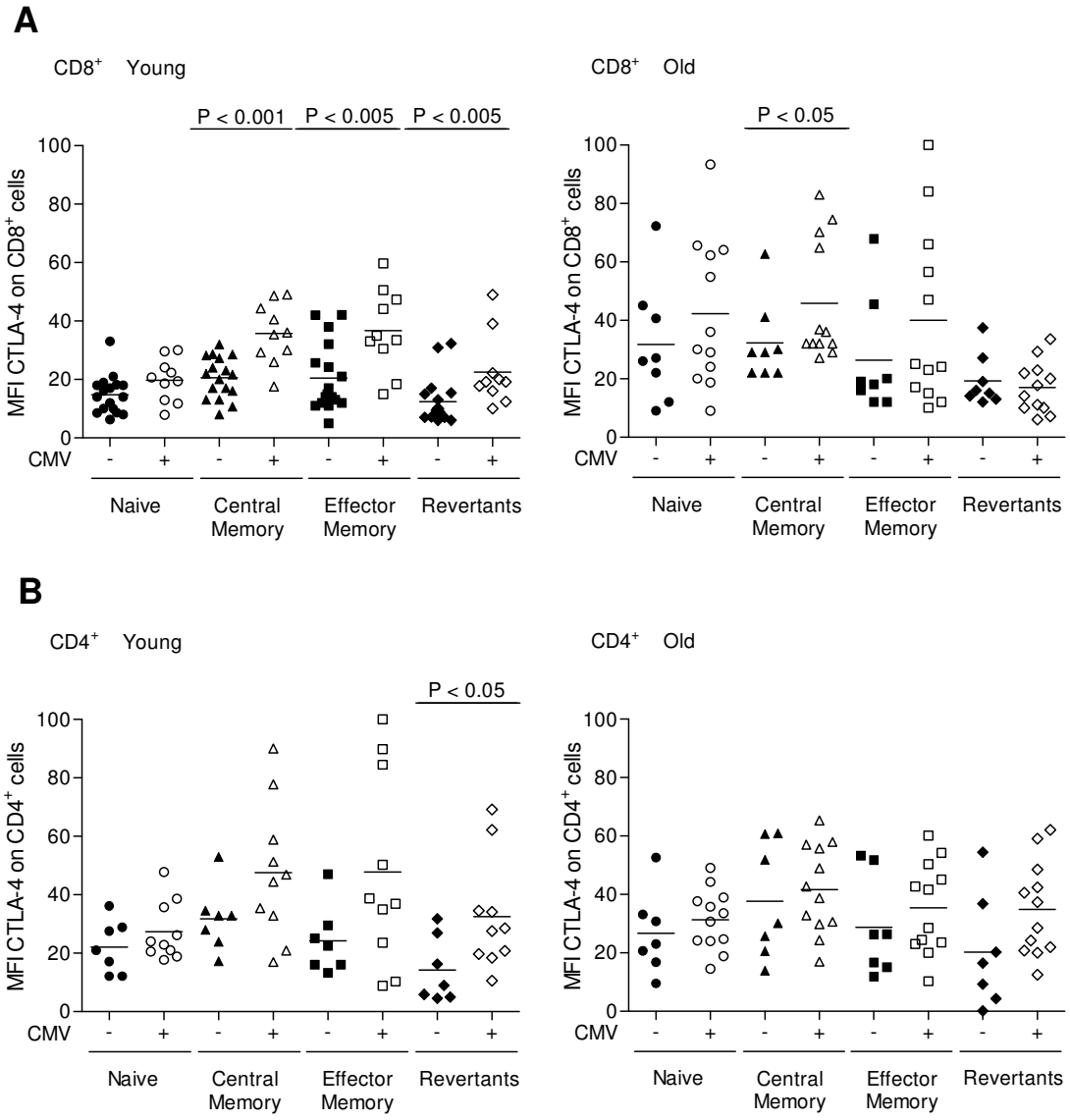


Figure 3.13 Differences in CTLA-4 expression stratified by the phenotypic markers CD27 and CD45RA in CMV$^+$ and CMV$^-$, old and young donors

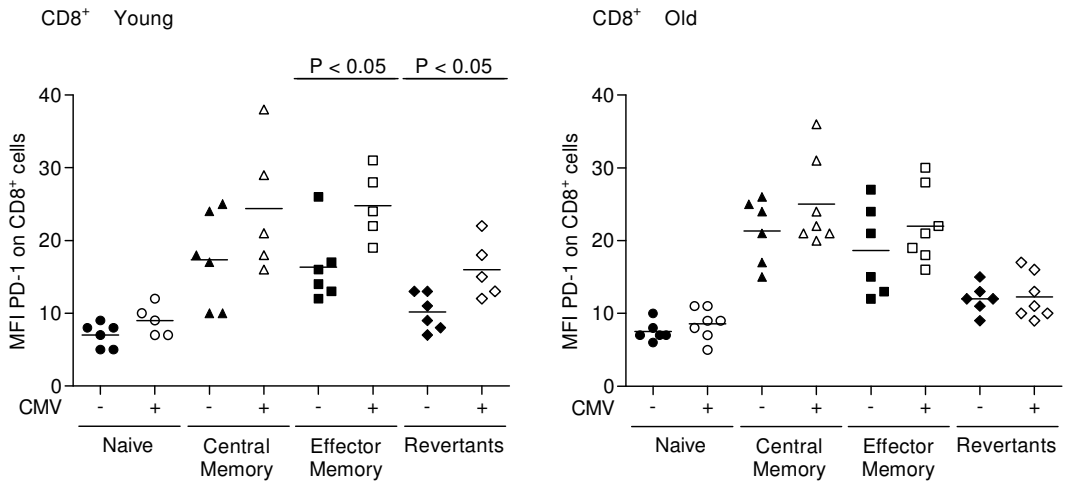
CTLA-4 expression on CD4$^+$ and CD8$^+$ T cells, as defined by CD27/CD45RA expression, determined as described in Fig 3.11 and 3.12, was compared between CMV$^-$ and CMV$^+$, old and young cohorts. **(A)** Pooled data comparing the expression of CTLA-4 staining in CMV$^-$ and CMV$^+$ donors on T cell subsets as defined by CD27 and CD45RA on CD8$^+$ or **(B)** CD4$^+$ T cells from **(left panels)** young and **(right panels)** old individuals. Young donors are defined as <35 years and old donors, >65. Filled symbols represent CMV negative individuals and open symbols signify CMV positive donors. Horizontal lines depict mean values. The P values were calculated using Mann-Whitney U-test. Significant differences in CTLA-4 subset expression between CMV$^-$ and CMV$^+$ donors are shown.

3.6.2 PD-1

PD-1 is expressed primarily on central and effector memory $CD8^+$ T cells both on CMV^+ and CMV^- donors independently of age. However, donors seropositive for CMV expressed higher levels of PD-1, which were significant amongst young individuals' effector and CD45RA-revertant memory subsets (Fig 3.14A left panel), but did not reach significance on $CD8^+$ T cells of old individuals at any differentiation stage (Fig 3.14A right panel). Similarly, the differing PD-1 expression pattern on $CD4^+$ T cells is observed independent of CMV status, amongst both young and old donors, and although CMV^+ donors express higher levels of PD-1, this does not reach significance at any phenotypic stage of young or old subjects (Fig 3.14B).

PD-1

A



B

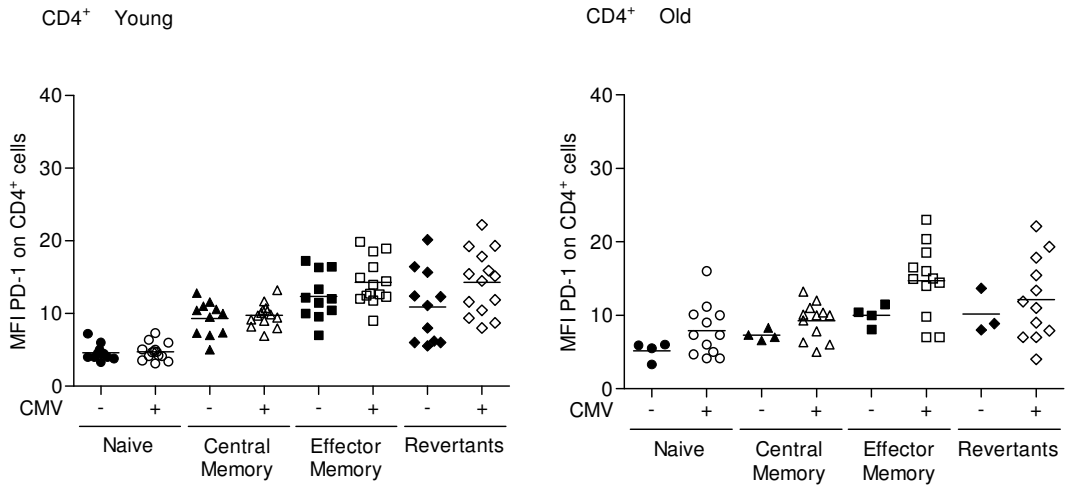


Figure 3.14. PD-1 expression correlated by CMV status in young and old donors on different CD8+ T cell differentiation stages in young and old donors

PD-1 expression on CD8+ and CD4+ T cell subsets, defined by CD27/CD45RA expression, was compared between CMV- and CMV+ age matched donors, as described in Fig 3.11 and 3.12. **(A)** Cumulative data showing the expression of PD-1 with respect to CD45RA and CD27 staining in CMV- versus CMV+ individuals amongst **(left panels)** young and **(right panels)** old donors on CD8+ and **(B)** CD4+ T cells. Filled symbols represent CMV negative individuals and open symbols signify CMV positive donors. Young donors are defined as <35 years and old donors, >65. Horizontal lines depict mean values. The P values were calculated using a Mann-Whitney U test. Significant differences in PD-1 between CMV- and CMV+ donors within a given subset are shown.

3.6.3 KLRG1

KLRG1 expression increases with differentiation on $CD8^+$ T cells both in CMV^+ and CMV^- donors from young and old cohorts (Fig 3.15). However, CMV^+ individuals exhibit significantly augmented KLRG1 expression, compared with their CMV^- counterparts, among the naïve $CD8^+$ T cells of young donors (Fig 3.15A left panel) and the central and effector memory $CD8^+$ subpopulations of old donors (Fig 3.15A right panel). In contrast, dependent on their CMV status, individuals exhibit a distinct pattern of KLRG1 expression on different $CD4^+$ T cell subpopulations. KLRG1 expression was greatest on CMV^- donors' effector memory subsets, but amongst CMV^+ individuals, CD45RA-revertant memory $CD4^+$ T cells expressed the highest levels (Fig 3.15B). This effect is most strikingly observed amongst the aged whose CMV^+ donors exhibit significantly higher levels of KLRG1 expression on their effector memory cells, compared with their CMV^- counterparts, but exhibit an increase of even greater magnitude on their CD45RA-revertant memory cells (Fig 3.15B right panel).

KLRG1

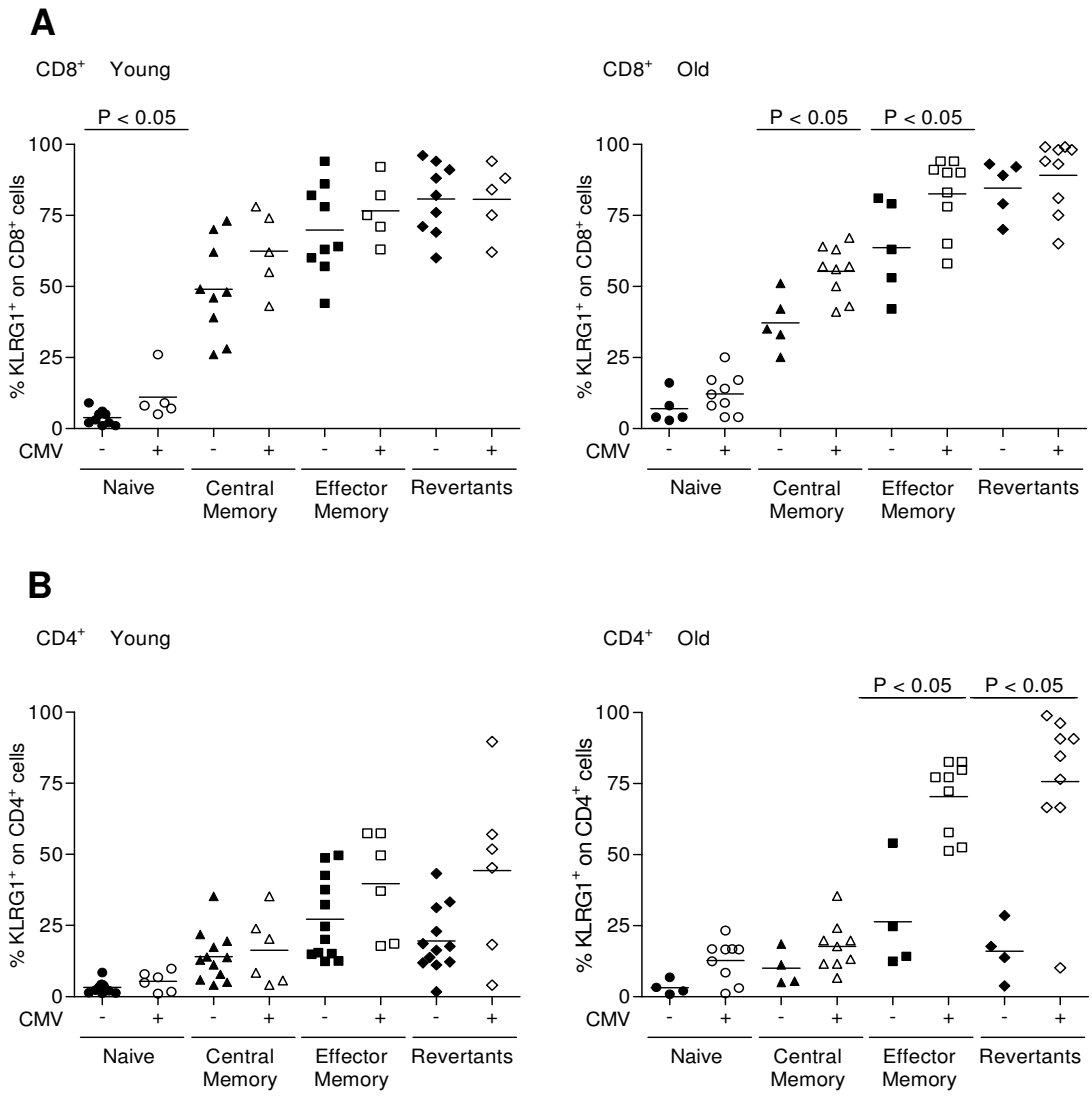


Figure 3.15. KLRG1 expression defined by differentiation status of CD8⁺ T cells in young and old donors with and without CMV

KLRG1 expression by different CD4⁺ and CD8⁺ T cell subsets, accomplished as described in Fig 3.11 and 3.12, was stratified by donor age and CMV status. **(A)** Cumulative data comparing KLRG1 expression with respect to CD45RA and CD27 in CD8⁺ and **(B)** CD4⁺ T cells of **(left panels)** young and **(right panels)** old donors. Filled symbols represent CMV negative individuals and open symbols signify CMV positive donors. Young donors are defined as <35 years and old donors, >65. Horizontal lines depict mean values. The P values were calculated using a Mann-Whitney U-test and only significant differences in PD-1 between CMV⁻ and CMV⁺ donors within a given subset are shown.

3.7 Characterisation of inhibitory receptor expression on CD27/CD28 defined subsets

3.7.1 CTLA-4 is expressed on early, PD-1 on intermediate and KLRG1 is expressed on late differentiated CD8$^+$ T cells

Utilising the alternative phenotypic markers CD27 and CD28 to distinguish different CD8$^+$ T cell stages of differentiation, CTLA-4 expression peaks on early differentiated CD8$^+$ T cells and diminishes with differentiation, with both young and old displaying similar patterns of expression (Fig 3.16A). PD-1 is similarly expressed on young and old individuals, both being maximally expressed on intermediate differentiation stages (Fig 3.16B). KLRG1 expression increases with differentiation on both the young and old but is significantly higher at each differentiation stage on old compared with young subjects (Fig 3.16C)

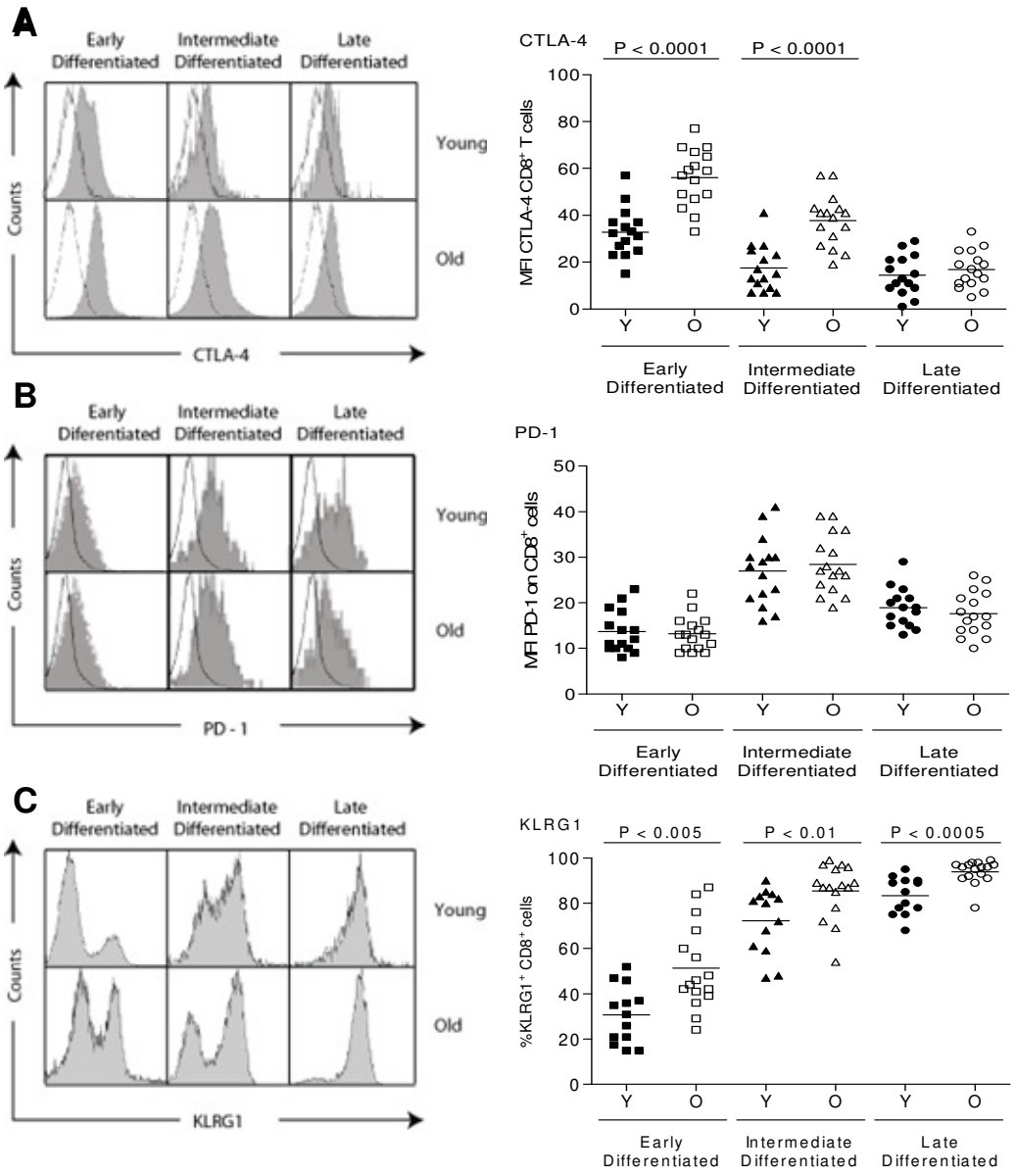


Figure 3.16. Comparison of inhibitory receptor expression on CD8+ T cells stratified by CD28/CD27 expression

CTLA-4, PD-1 and KLRG1 expression was quantified as described in Fig 3.07, 308 and 3.09, respectively, on CD8+ T cells subsets as defined by CD27 and CD28 staining as detailed in Fig 3.04. **(A, left panel)** Histograms depicting CTLA-4, **(B, left panel)** PD-1 and **(C, left panel)** KLRG1 expression on CD8+ T cell subsets from a representative young, Y <35yrs and old , O >65, donor. Filled plots indicate inhibitory receptor staining whereas unfilled plots represent isotype controls. **(A, right panel)** Pooled data comparing CTLA-4, **(B, right panel)** PD-1 and **(C, right panel)** KLRG1 expression at different CD8+ T cell differentiation stages in young and old individuals. Horizontal lines depict mean values. All the CTLA-4 and PD-1 data sets passed a D'Agostino & Pearson omnibus normality test and their P values were calculated using a Student's t-test. However, not all the KLRG1 data sets followed a Gaussian distribution and thus a Man-Whitney U test was used to assess P values and only significant differences between young and old donors are shown

3.7.2 Alterations in inhibitory receptor expression as defined by the phenotypic markers CD27 and CD28 on CD4$^+$ T cells on both young and old donors

Inhibitory receptors display an alternative expression profile on CD4$^+$ T cells with CTLA-4 being similar in the young and old and peaking on intermediate differentiated rather than early differentiated subsets (Fig 3.17A). PD-1 expression is greatest amongst the intermediate and highly differentiated CD4$^+$ T cells (Fig 3.17B). KLRG1 increases with differentiation and is higher in old compared to young individuals at each differentiation stage (Fig 3.17C), as observed among CD8$^+$ T cells, however altogether CD4$^+$ T cells express KLRG1 at comparably lower levels.

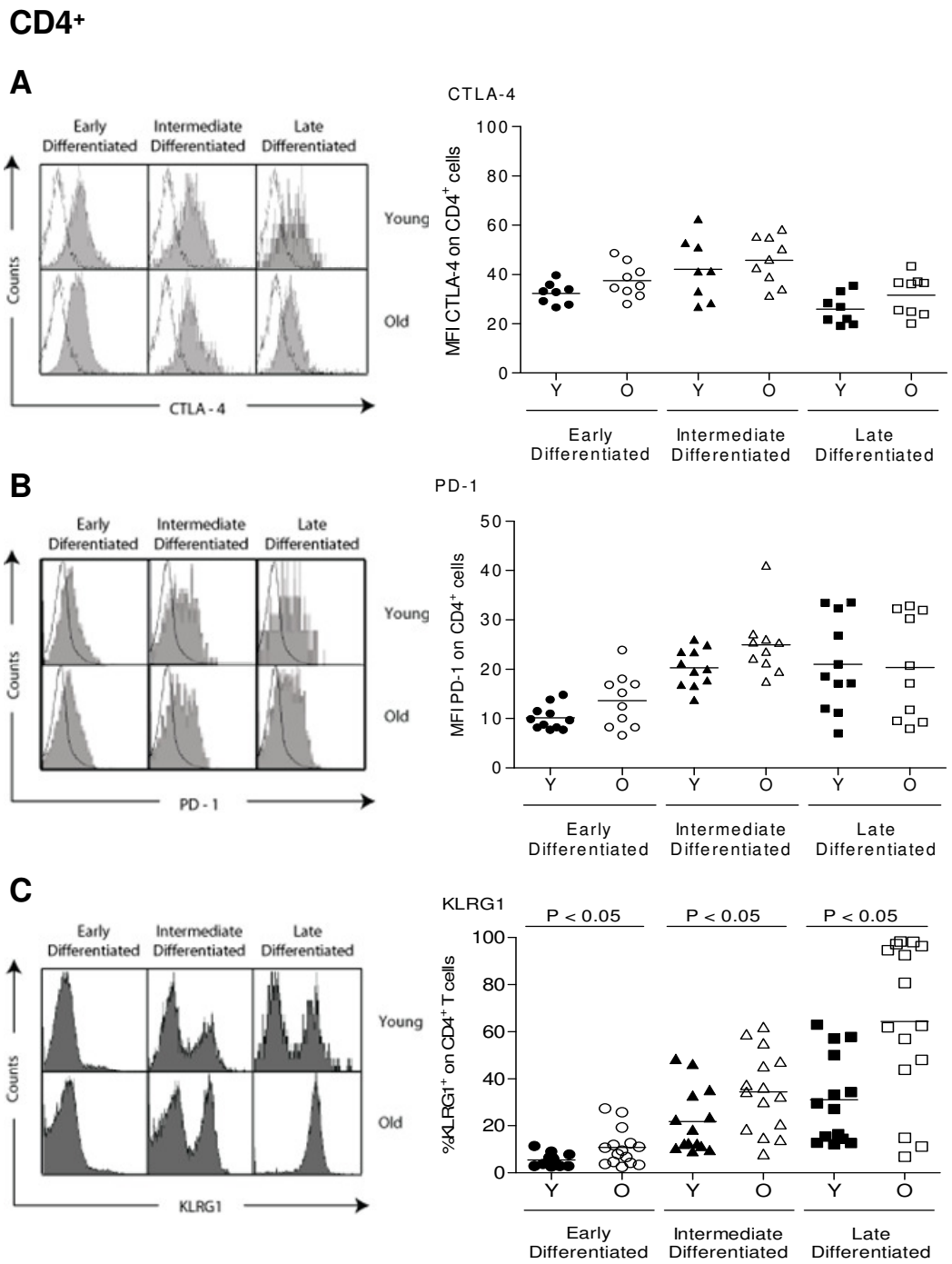


Figure 3.17. Inhibitory receptor expression on different CD4+ T cell differentiation stages correlated by age of the donors

Inhibitory receptor levels were quantified on CD4+ CD27/CD28 subsets by flow cytometry as detailed in Fig 1.10. **(A, left panel)** Histograms depicting the CTLA-4, PD-1 and KLRG1 expression on the different CD4+ T cell differentiation stages of a representative young and an old donor **(A, right panel)** Cumulative data depicting the expression of CTLA-4, **(B right panel)** PD-1 and **(C, right panel)** KLRG1 with respect to CD27 and CD28 staining amongst young (Y, <35 years) and old (O, >65) donors. Horizontal lines depict mean values. The P values were calculated using a Mann-Whitney U test. Significant differences between young and old donors within a given subset are shown.

3.7.3 CD28 expression on CD27/CD45RA defined $CD4^+$ and $CD8^+$ T cell subsets

To account for discrepancies in the inhibitory receptor expression data on different T cell subpopulations using CD27/CD45RA versus CD27/CD28 markers, it was attempted to define the degree of overlap between the subsets identified using these two sets of markers. This was achieved by characterising CD28 expression on $CD8^+$ and $CD4^+$ T cell subpopulations, as defined by CD27/CD45RA expression. CD28 is expressed on approximately half of all $CD8^+$ T cells with expression being greatest on central memory T cells followed by naïve with low levels on effector memory CD45RA re-expressing memory T cells, (Fig 3.18A, representative example; Fig 3.18C, pooled data). In contrast, $CD4^+$ T cells exhibit very high levels of CD28 expression with near universal expression on naïve and central memory cells. A slight reduction was observed in effector memory cells and low levels were only found on the CD45RA-revertant memory subset, (Fig 3.18B, representative example; Fig 3.18D, pooled data).

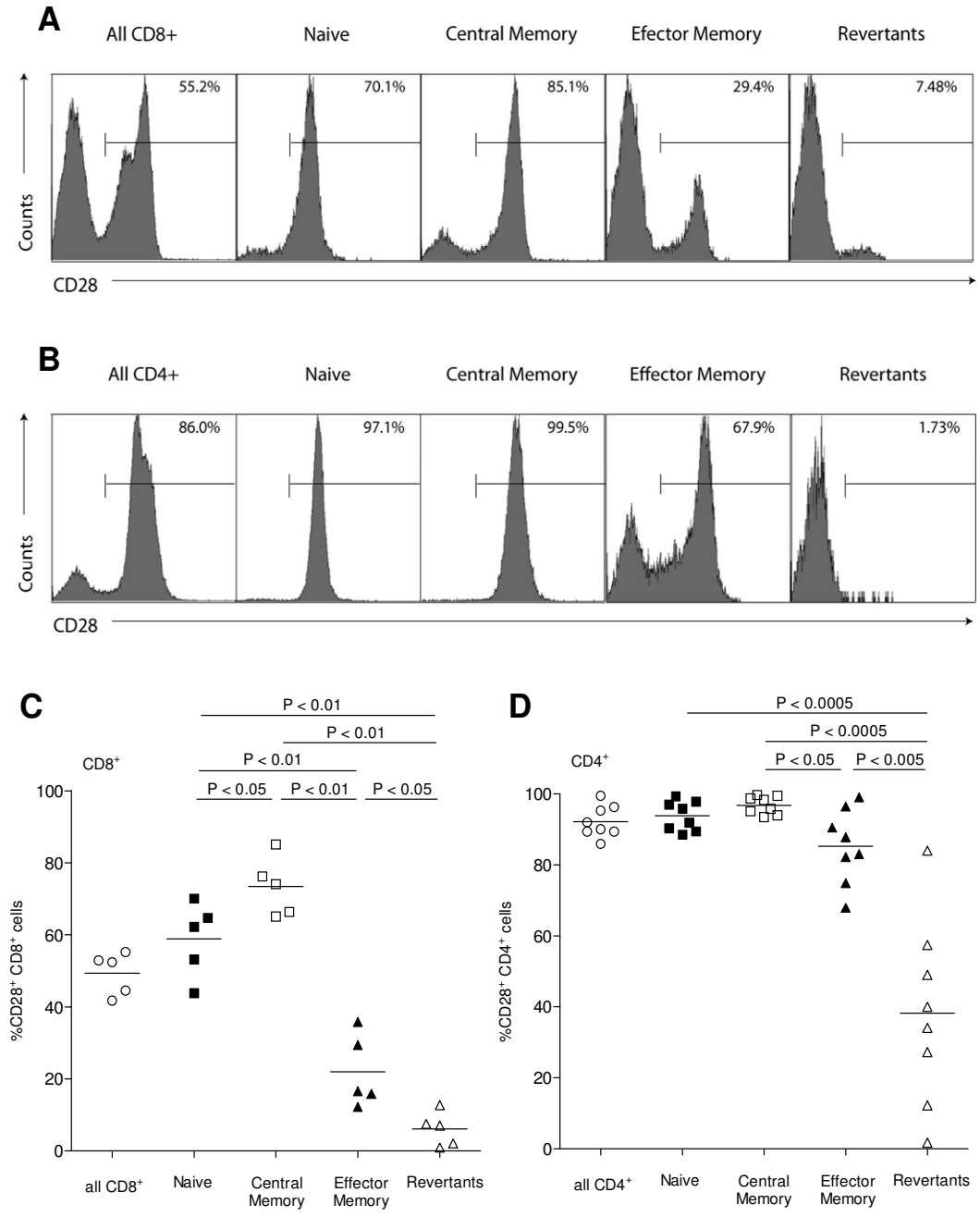


Figure 3.18. The expression of CD28 at different T cell differentiation stages.

PBMCs were stained with anti- CD27-FITC, CD28-PE, CD8-PerCP, CD45RA-APC and CD4-PE-Cy7. **(A)** Representative histograms for one donor are shown with the mean expression of CD28 indicated for each of the $CD8^+$ or **(B)** $CD4^+$ T cell subsets as defined by CD27/CD45RA. **(B)** Scatter graphs showing the expression of CD28 on each $CD8^+$ and **(C)** $CD4^+$ T cell subset for all donors ($CD8^+$, n=5; $CD4^+$ n=8). Horizontal bars represent mean values. P values generated using a Mann-Whitney U test. Significant differences between subsets displayed.

3.8 Determination of the Functional Significance of Inhibitory Receptors in T cell Ageing

To define whether these observed inhibitory receptor expression changes are causative of age onset immune decline, the inhibitory receptor signalling pathway was interrupted by antibody blockade to determine whether age-associated immune cellular dysfunction could be reversed. PD-1 and KLRG1 were blocked using antibodies specific for their major ligands PD-L1 & PD-L2 and E-cadherin, respectively, whereas CTLA-4 was blocked directly using CTLA-4 blocking antibodies. Functional exhaustion comprises a spectrum of defects with proliferative impairment being a key feature, occurring when other functions are intact, and becoming increasingly impaired across the exhaustion gradient (Freeman et al., 2006) and furthermore, maintenance of high proliferative potential amongst chronic virus specific $CD8^+$ T cells is associated with robust protective immunity (Migueles et al., 2002). Therefore proliferation was used as a functional readout to monitor reversed immune dysfunction.

3.8.1 Inhibitory Receptor Blockade Significantly Augments the $CD8^+$ T cell Proliferative Responses of Young Donors

To measure the effects of inhibitory receptor blockade on proliferation, MACS purified $CD8^+$ or $CD4^+$ T cells from young and old individuals were stimulated for 3 days with anti-CD3 in the presence of irradiated autologous APCs, which functioned as a source of costimulatory molecules. Either an inhibitory receptor blockade or its relevant isotype control antibody was added to the purified cells and [^{3}H]-thymidine

incorporation was measured over the last 18hours (Fig 3.19). A representative example of the purity of $CD8^+$ T cells obtained from MACS separation is displayed in figure 3.20. CTLA-4, PD-1 and KLRG1 blockade induced significant increases in proliferation compared to relevant isotype controls in $CD8^+$ T cells of the young (Fig 3.21A, representative example; Fig 3.21B left panel) and old donors (Fig 3.21B right panel). However, although blocking inhibitory receptors could augment the proliferative responses of $CD4^+$ T cells this was of diminished significance compared with that observed for $CD8^+$ T cells (Fig 3.22A, representative example; Fig 3.22B, cumulative data).

These data show that blocking inhibitory receptors significantly increased cellular proliferation in response to anti-CD3 stimulus. However, the proliferative increases observed for $CD8^+$ T cells were smaller in older individuals (Fig 3.21, $CD8^+$ 0.8-9.7 fold increase; Fig 3.22, $CD4^+$ 0.7-2.2) compared with the young (Fig 3.21, $CD8^+$ 1.0-2.9; Fig 3.22, $CD4^+$ 0.7-1.4). This would suggest that the proliferative hypo-responsiveness of $CD8^+$ T cells from old individuals to antigenic stimuli cannot be completely explained by the age related increase in inhibitory receptor expression on $CD8^+$ T cells. No additive or synergistic effects were observed following simultaneous blockade of multiple inhibitory receptors on either $CD4^+$ or $CD8^+$ T cells proliferation. However, what is strikingly apparent from these data is that there is a large degree of heterogeneity in the $CD8^+$ T cell proliferative response of different individuals to inhibitory receptor blockade, consistent with the heterogeneous proliferative responses to cognate antigen observed following blocking of PD-1 and CTLA-4 (Kaufmann et al., 2007).

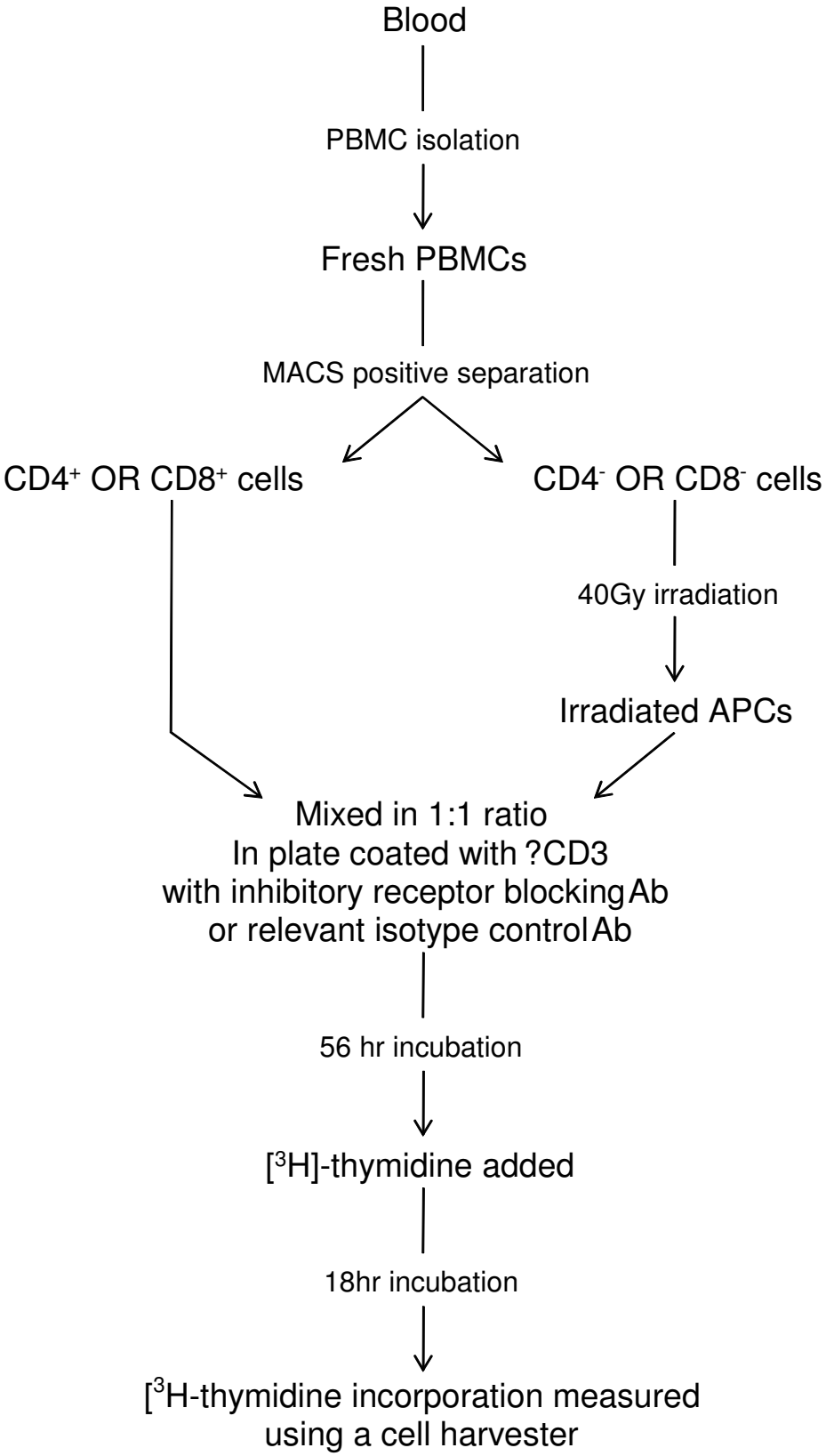


Figure 3.19 Schematic illustrating the experimental procedure for detecting the effect of inhibitory receptor blockade on anti-CD3 induced $CD4^+$ and $CD8^+$ T cell proliferation.

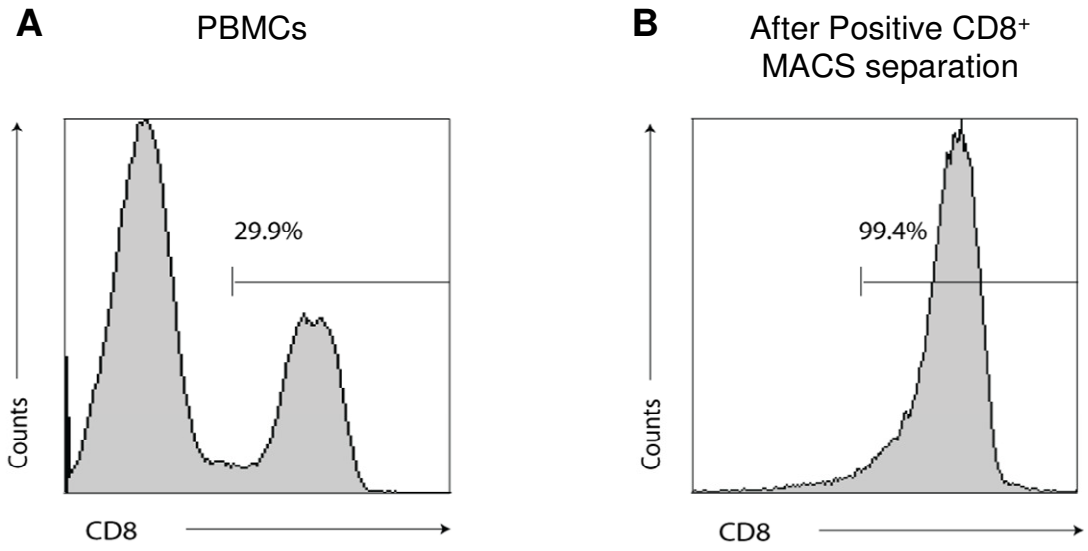


Figure 3.20 Purity of cells obtained from MACS positive separation.

$CD8^+$ T cells, separated from PBMCs using MACS positive isolation kit, were stained with anti-CD8-PerCP. Representative histograms illustrating the proportion of lymphocytes that are $CD8^+$ **(A)** before and **(B)** after MACS positive separation

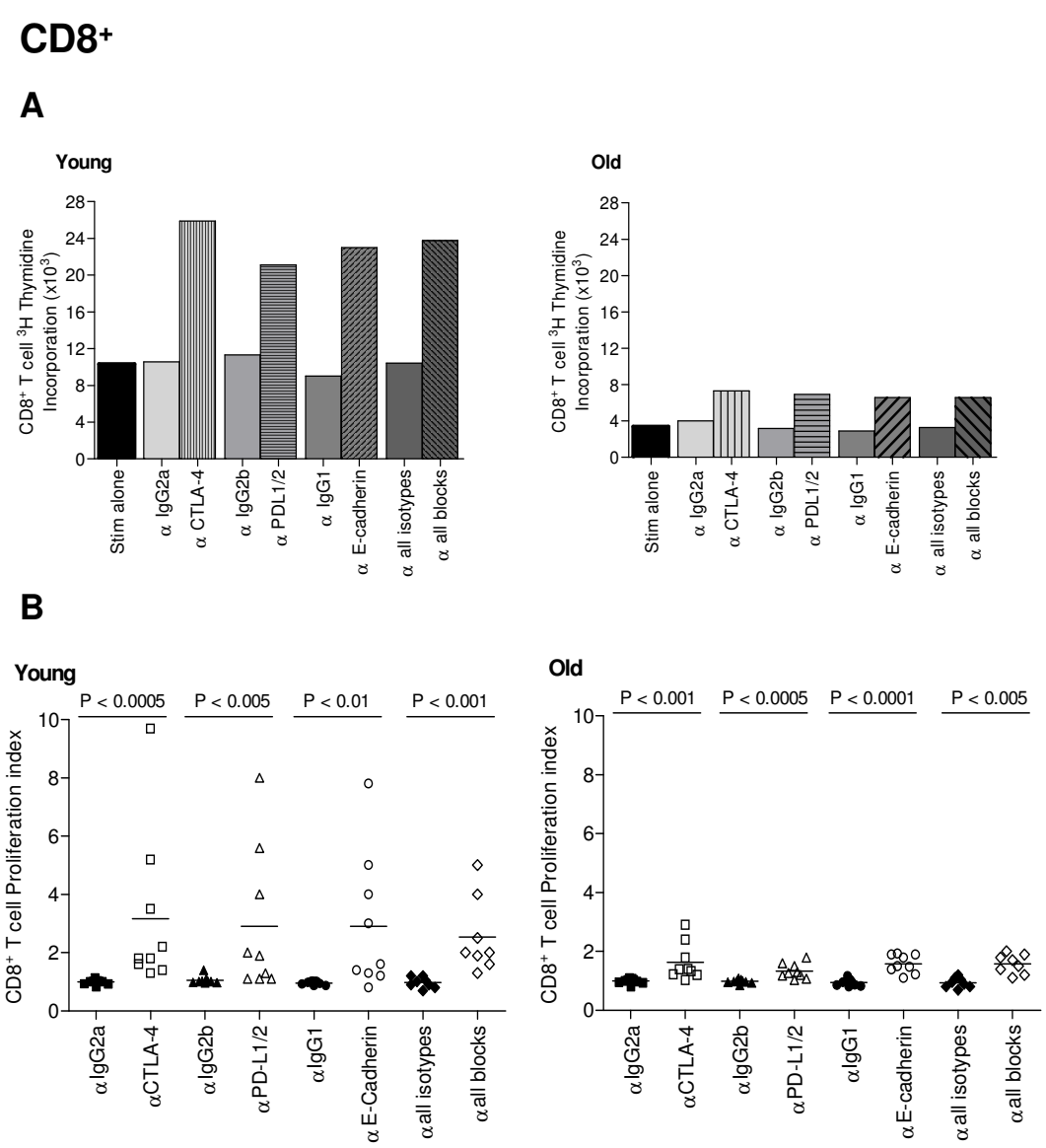


Figure 3.21. Effects of inhibitory receptor blockade on CD8+ T cell proliferation in young and ld individuals.

CD8+ T cells, isolated from the PBMCs of young (<35yrs, n = 9) and (>65 years, n = 9) old donors using MACS sorting, were stimulated for 72 hours with anti-CD3 and autologous irradiated APCs in the presence of anti-CTLA-4, PD-L1/2 and E-cadherin blocking antibody or the relevant isotype control. Cellular proliferation was measured by thymidine incorporation over the last 18hours. **(A)** Representative example changes in thymidine incorporation values following inhibitory receptor blockade versus the relevant isotype control of the CD8+ T cells of a **(left panel)** young and **(right panel)** old person. **(B)** Pooled data comparing proliferation of CD8+ T cells from **(left panel)** young and **(right panel)** old individuals following inhibitory receptor antibody blockade. Proliferation index was calculated by determining the ratio between cells proliferating in the presence of anti-CD3 stimulation versus inhibitory receptor blockade. The P values were calculated using a Mann-Whitney U test. Significant differences between inhibitory receptor blocks and their isotype controls are displayed.

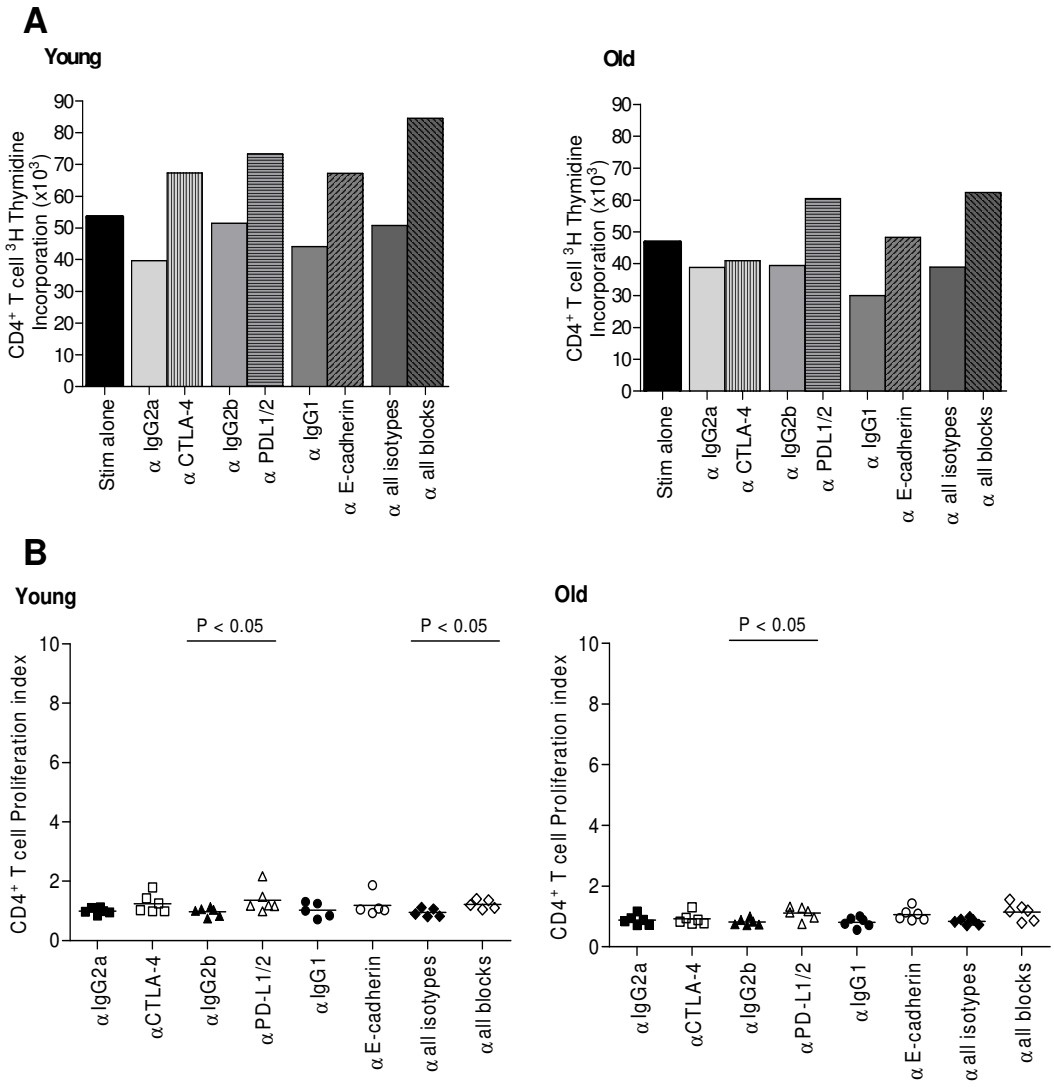


Figure 3.22. Effects of inhibitory receptor blockade on CD4+ T cell proliferation in young and old individuals

MACS separated CD4+ T cells were stimulated with anti-CD3 and autologous irradiated APCs for 72 hours in the presence of inhibitory receptor blocking antibodies or the relevant isotype control and the amount of thymidine incorporated over the final 18 hours was taken as a measure of CD4+ T cell proliferation. **(A)** Bar chart illustrating changes in thymidine incorporation following inhibitory receptor blockades versus the isotype controls in a representative **(left panel)** young and **(right panel)** old donor. **(B)** Cumulative data comparing the effects of blocking inhibitory receptors compared with their relevant isotype controls on the proliferation of CD4+ T cells from **(left panel)** young and **(right panel**) old donors. Horizontal bars depict mean values. Proliferation index was calculated by determining he ratio between cells proliferating to anti-CD3 stimulation with inhibitor receptor blockade in comparison with the addition of their relevant isotype control. The P values were calculated using a Mann-Whitney U test. Significant differences between inhibitory receptor blocks and their isotype controls are displayed.

3.8.2 The proliferative deficit of highly differentiated CD8$^+$ T cells can be significantly reversed upon blocking KLRG1 interactions with its ligand

The functional relevance of inhibitory receptor expression at different T cell differentiation stages was then investigated by performing inhibitory receptor blocking experiments as described above but using purified CD8$^+$ T cell subsets rather than whole CD8$^+$ T cells. Blocking PD-L, CTLA-4 and E-cadherin significantly augmented the proliferative responses of early and intermediate differentiated CD8$^+$ T cell subsets (Fig 3.23 B-D). However, only E-cadherin blockade was able to significantly increase the proliferation of highly differentiated CD8$^+$ T cells (Fig 3.23A, representative example; Fig 3.23D, pooled data).

CD8+

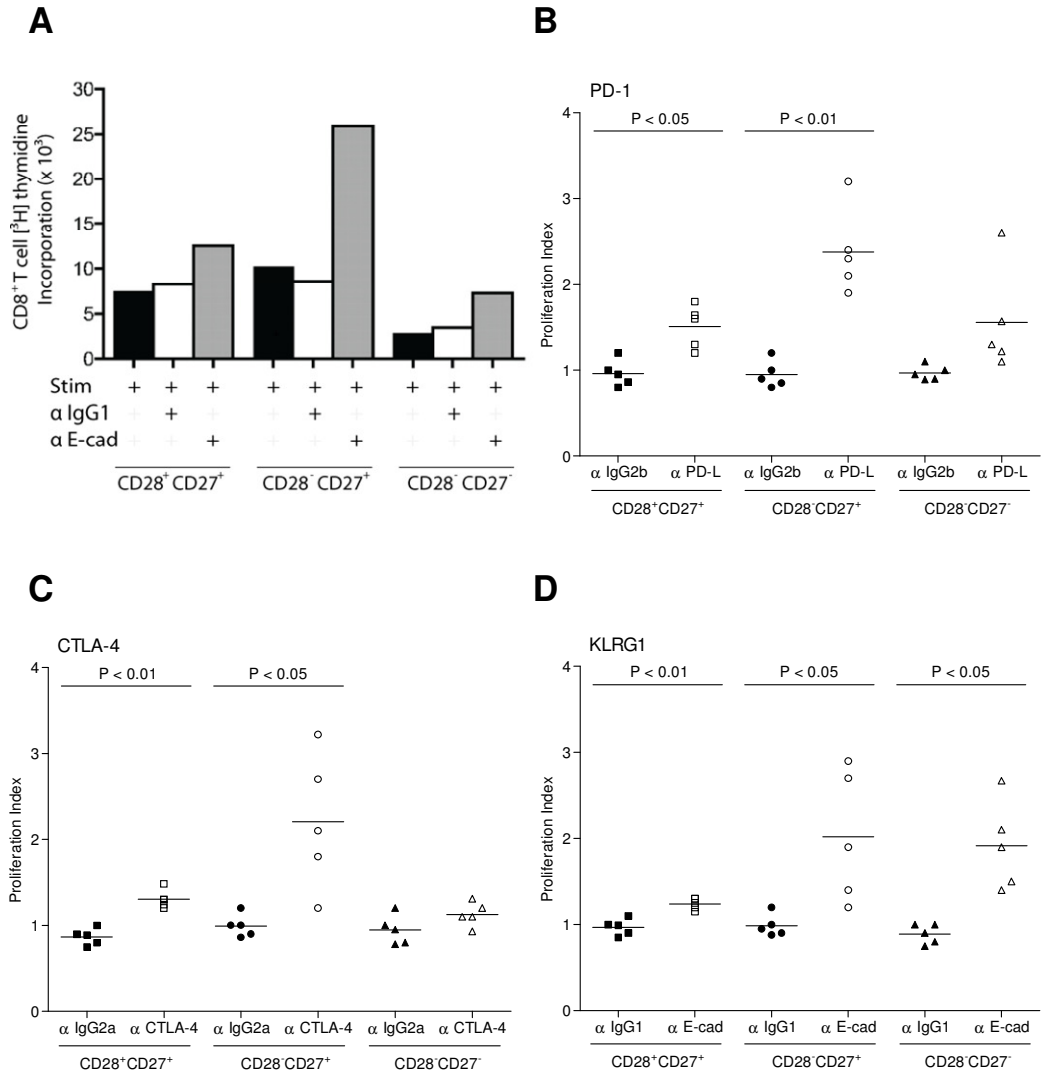


Figure 3.23. Effects of inhibitory receptor blockade on proliferative responses of CD8+ T cells at different stages of their differentiation.

MACS separated CD8+ T cells subsets, identified on the basis of their CD27/CD28 expression (detailed in figure 3.02), from 5 young donors were stimulated with anti-CD3 for 72 hours in the presence of autologous irradiated APCs and either inhibitory receptor blocking antibody or their relevant isotype control. Thymidine incorporation over the last 18 hours was taken as a measure of CD8+ T cell subset proliferation. **(A)** Representative bar chart illustrating proliferative responses of CD8+ CD27/CD28 subsets in response to E-cadherin blockade as compared to isotype control or stimulation alone. **(B)** Pooled data comparing the effects of anti-PD-L **(C)** anti-CTLA-4 and **(D)** anti- E-cadherin blocking antibodies on different CD8+ T cell subsets, as defined by CD27/CD28 expression. Horizontal bas represent mean values. Proliferation index was calculated by determining the ratio between cells proliferating to anti-CD3 stimulation with inhibitor receptor blockade in comparison with the addition of their relevant isotype control. The P values were calculated using a Mann-Whitney U test. Significant differences between inhibitory receptor blocks and their isotype controls are displayed.

The finding that KLRG1 mediated signalling contributes to the attenuated proliferative responses of highly differentiated $CD8^+$ T cells was further extended by other members of the Akbar group who elucidated the precise role of this molecule in mediating other dysfunctions of this $CD8^+$ T cell population (Table 3.01). As well as reduced proliferative capacity, these cells have several characteristic defects including defective Akt phosphorylation at Ser^{473} (Plunkett et al., 2007), which could be augmented upon blocking KLRG1/E-cadherin interactions (Henson et al., 2009). However, their characteristic impaired telomerase induction (see Chapter 5) and IL-2 synthesis capacity were not reversible upon E-cadherin blockade (Henson et al., 2009). Extending these studies to aged donors revealed that although blocking E-cadherin could still significantly reverse the defective Akt phosphorylation of their highly differentiated $CD8^+$ T cells, this was accompanied by a proliferative increase of much reduced magnitude compared to that observed in the young. This suggests that other age related defects contribute towards the characteristic dysfunctional proliferative responses of highly differentiated $CD8^+$ T cells in old subjects.

Features of highly differentiated CD8+ T cells	Reversible upon E-cadherin blockade?
Reduced proliferative capacity	
Poor capacity for IL-2 synthesis	
Defective Akt synthesis after activation	
Reduced telomerase activity	

Table 3.01 Functional roles for KLRG1 in highly differentiated human CD8+ T cells.

Summary of data generated by the Akbar group, illustrating key features of highly differentiated CD8+ T cells and which of these can be at least partially reversed upon blocking interactions of KLRG1 with its ligand E-cadherin

KLRG1 signaling induces defective Akt (Ser473) phosphorylation and proliferative dysfunction of highly differentiated CD8+ T cells. Henson,S.M.; Franzese,O.; **Macaulay,R**.; Libri,V.; Azevedo,R.I.; Kiani-Alikhan,S.; Plunkett,F.J.; Masters,J.E.; Jackson,S.; Griffiths,S.; Pircher,H.P.; Soares,M.V.; Akbar,A.N. Blood. 2009 Apr 30.

3.9 Discussion

This chapter addresses the potential role of immune inhibitory receptors in age onset immune decline. Data presented here demonstrate that the immune inhibitory receptors CTLA-4, PD-1 and KLRG1 can be upregulated by both CMV and age in total $CD4^+$ and $CD8^+$ T cells and also at different stages of their differentiation. Antibody blocking studies reveal that although none of these receptors directly mediates the general $CD8^+$ T cell hypo-responsiveness seen during ageing, KLRG1 signalling contributes towards the characteristic dysfunctional proliferative responses and Akt phosphorylation of highly differentiated $CD8^+$ T cells in younger subjects.

These results confirm previous reports that CMV infection can be associated with global changes to the hosts peripheral T lymphoid pool, where CMV is said to leave a fingerprint of infection (van de Berg et al., 2008), being associated with lymphocyte phenotype alterations very similar to those published as age-associated (Weinberger et al., 2007). Similarly, rapid alterations are observed following primary infection of renal transplant recipients with CMV, which is suggestive of CMV playing a causative role in these changes (van de Berg et al., 2008; van de Berg et al., 2010), that have been estimated to accelerate the physiological decline by 35 to 40 years (Chidrawar et al., 2009; Northfield et al., 2005). An inverted CD4:CD8 ratio is an important component of the IRP, which results from the increased prevalence of $CD27^-CD28^-CD8^+$ T cells, whose numbers are augmented in those infected with CMV (Pawelec and Derhovanessian, 2010). Indeed, CMV infection has been

suggested to influence all parameters of the IRP with the possible exception of low B cell numbers (Derhovanessian et al., 2010).

The striking features of this data, in how ageing affects $CD4^+$ and $CD8^+$ T cell compartments differently, is reflected in $CD4^+$ T cells being more resistant to CMV and age-associated phenotypic changes than $CD8^+$ T cells, concurrent with studies in aged humans (Weinberger et al., 2007; Czesnikiewicz-Guzik et al., 2008; Kovaiou et al., 2005; Koch et al., 2007) and CMV infected humans (Weinberger et al., 2007; Northfield et al., 2005; Pourgheysari et al., 2007; Almanzar et al., 2005; Looney et al., 1999; Gratama et al., 1987). This may reflect the more clonal nature of $CD8^+$ T cell responses (Maini et al., 1999), in that following antigenic challenges, $CD8^+$ T cells exhibit more rapid expansion kinetics, producing larger numbers of responding effector cells and memory cells (Beverley, 2008). Additionally, this difference may also be accounted for by their different lifespans (Aw et al., 2007), apoptotic sensitivities (Pawelec et al., 2002), gene expression instabilities (Czesnikiewicz-Guzik et al., 2008) or thymic selection biases (Czesnikiewicz-Guzik et al., 2008).

T cell inhibitory receptor expression characterisation with respect to age revealed that only CTLA-4 and KLRG1 on $CD8^+$ T cells underwent significant expression increases with age, consistent with previous reports (Thimme et al., 2005; Czesnikiewicz-Guzik et al., 2008; Fann et al., 2005; Ouyang et al., 2003a). The observation that CTLA-4 shows no age related upregulation on $CD4^+$ T cells contrasts with a previous report by Leng et al. (Leng et al., 2002a). However, this group did not stratify their data on the basis of CMV status, whereas data presented here shows

CTLA-4 upregulation on CMV positive donors. Therefore, the increased CTLA-4 expression observed by Leng *et al* may be a consequence of age-associated increases in CMV prevalence (Stowe et al., 2007; McVoy and Adler, 1989; Looney et al., 1999; Dowd et al., 2009).

This lack of any large age-associated inhibitory receptor changes amongst $CD4^+$ T cell subsets, correlates with the lack of functional impairment observed in $CD4^+$ T cell subsets of old versus young donors (Kovaiou et al., 2005), suggesting that age-associated changes in the function of the $CD4^+$ T cell compartment may primarily be a consequence of quantitative alterations in $CD4^+$ subsets to more differentiated cells.

Data presented here reveal that CMV^+ individuals express significantly higher levels of PD-1, CTLA-4 and KLRG1 on their $CD4^+$ and $CD8^+$ T cells, consistent with the well documented role of CMV infection in inducing dramatic phenotypic changes to the total T cell pool, in otherwise healthy individuals (as described earlier). These changes may reflect a combination of CMV associated immune activation, T cell differentiation and alterations to the host cytokine profile.

Amongst $CD8^+$ T cells, CTLA-4 expression peaks in naïve and central memory stages of differentiation, PD-1 is maximal amongst central and effector memory and KLRG1 peaks in the CD45RA-revertant memory differentiation stages. An alternate profile emerges within the $CD4^+$ T cell compartment, with PD-1 and KLRG1 expression peaking on highly differentiated and CTLA-4 maximal at intermediate differentiation stages. These data are consistent with intracellular CTLA-4 stores being present in

memory T cells, enabling a greater and more rapid induction of expression compared with naïve cells, who have no such stores and synthesise CTLA-4 *de novo* (Jago et al., 2004). Moreover, PD-1 and KLRG1 expression have previously been demonstrated to peak on intermediate (Sauce et al., 2007a; Petrovas et al., 2006) and highly differentiated (Voehringer et al., 2002; Thimme et al., 2005; Henson et al., 2009) $CD8^+$ T cells, respectively. This gives rise to the novel idea that as $CD4^+$ and $CD8^+$ T cells differentiate they utilise different inhibitory receptors, which may make distinct contributions to the function of different T cell subsets. Furthermore, T cells specific for different chronic viruses occupy distinct differentiation stages (van Leeuwen et al., 2006a; Kuijpers et al., 2003), and may thus induce unique sets of inhibitory receptors that differentially impair antiviral immunity. This idea may have particular relevance in the exploration of inhibitory receptor blocking therapies as immuno-therapeutic regimens to augment chronic viral treatments and vaccination strategies. Indeed, blocking CTLA-4 and PD-1 have been explored as a strategy to augment vaccine responses against tumours and chronic pathogens (Yuan et al., 2011; Curran and Allison, 2009; Song et al., 2011; Ha et al., 2008). Moreover, increasing the efficacy of vaccinations, particularly influenza, for aged individuals would have an enormous impact on their health and well-being.

The age related upregulation of CTLA-4 on $CD8^+$ T cells was shown not to be a consequence of differentiation but reflects CTLA-4 upregulation, particularly on naïve cells, with age. In contrast, KLRG1 expression experiences a large and dramatic increase with differentiation on $CD8^+$ T cells and although age-associated upregulation on some subsets are observed, these are comparatively small in

magnitude, suggesting that KLRG1 expression upregulation with age on $CD8^+$ T cells is primarily a consequence of the increased prevalence of increasingly differentiated cells.

Following simultaneous TCR engagement, CTLA-4, PD-1 and KLRG1 all oppose TCR/CD28 mediated signalling and may thus contribute to setting a threshold for stimulation. Indeed, PD-1 and CTLA-4 appear to be most effective at inhibiting responses to suboptimal stimuli, such as self-antigens, whereas potent responses against microbial antigens are relatively unaffected (Abbas and Sharpe, 2005). Therefore, their increased expression may contribute towards the characteristic T cell hypo-responsiveness of aged individuals. The functional significance of inhibitory receptor upregulation is highlighted by the pathogenesis of HIV which, rather than being a consequence of direct viral cytopathic effects, has been suggested to reflect chronic immune activation (Appay et al., 2005), mediated in part through inhibitory receptor upregulation (Leng et al., 2002b).

However, inhibitory receptor blockade on $CD8^+$ T cells from old donors failed to augment their proliferative responses to a polyclonal T cell stimulus, to a level equivalent to those observed in young, which concurs with a recent report in mice (Lages et al., 2010). Moreover, highly differentiated $CD27^-CD28^-CD8^+$ T cells display defective proliferative responses and telomerase induction, possess short telomeres and are said to be close to replicative senescence (Plunkett et al., 2007). They also express high levels of KLRG1 and blocking interactions with its ligand

reversed these characteristic dysfunctions in proliferation and Akt phosphorylation, representing the first demonstration of a functional role of KLRG1 in primary human $CD8^+$ T cells (Henson et al., 2009). However, KLRG1 blockade in aged individuals resulted in a proliferative enhancement of these highly differentiated $CD8^+$ T cells of reduced magnitude compared with young individuals. These data suggest the proliferative hypo-responsiveness of aged $CD8^+$ T cells involve additional mechanisms independent of CTLA-4, PD-1 and KLRG1 signalling.

The inability of inhibitory receptor blockade to reinvigorate the general $CD8^+$ T cell hypo-responsiveness of aged individuals combined with the findings that expression of CTLA-4, PD-1 and KLRG1 on $CD8^+$ and $CD4^+$ T cells is more consistently correlated with CMV status than ageing. This leads to the idea that inhibitory receptors may play a role in immunosenescence by contributing to the dysregulation of CMV specific immune responses, which constitute a major force driving T cell immunosenescence (as discussed in section 1.3.2.2). This hypothesis is investigated in the next chapter.

4 Role of inhibitory receptors in mediating the dysregulated T cell response to CMV

4.1 Introduction

Replicative senescence is not just associated with old age but can be observed in relatively young individuals who have been subjected to a high antigenic load (as described in section 1.3.2). In particular, the ubiquitous and relatively innocuous herpesvirus CMV appears to act as the dominant chronic stressor (as described in section 1.4). Indeed, the magnitude of the immune response directed against CMV undergoes a progressive age related expansion and the increasing amount of immune resources dedicated to controlling CMV with advancing age may be a major contributor to age onset immuno-decline (as described in section 1.4.3.3.2). This so-called 'memory inflation' is especially prominent in the $CD8^+$ T cell compartment, which accumulates large dysfunctional CMV epitope-specific T cell expansions, often comprised of a small number of clones that have undergone extensive antigen driven proliferation (Shin et al., 2007). Indeed, it has been suggested that lifelong persistent CMV reactivation may drive clonal exhaustion of the most efficient and specific T cells so that an increased number of suboptimal cells are required to control virus infection (Akbar and Fletcher, 2005). Therefore, reinvigorating CMV specific $CD8^+$ T cell functions could allow better control of CMV infection and thus impair memory inflation and the development of immunosenescence. Indeed, mathematical models have demonstrated that decreasing antigenic burden leads to a considerable increase in the resistance to immunosenescence (Romanyukha and Yashin, 2003). This has

been supported by two studies illustrating that the treatment of an Ethiopian population for intestinal parasites (Kassu et al., 2003) and migration of Ethiopians to Israel (Kalinkovich et al., 1998), an environment with a comparatively low antigenic burden, is associated with a reversal of immune parameters to that of much younger individuals. In this chapter the role of inhibitory receptors in mediating these CMV specific $CD8^+$ T cell dysfunctions is investigated.

4.2 Characterisation of CMV specific $CD8^+$ T cells

4.2.1 Determination of donor CMV status

Purified PBMCs were either left unstimulated, as a negative control (Fig 4.01A), stimulated with superantigen SEB, as a positive control, (Fig 4.01B) or stimulated with CMV lysate. The $CD4^+$ T cells of donors who were identified as CMV seropositive significantly induced IFNγ production (Fig 4.01C), whereas CMV seronegative donors failed to secrete IFNγ (Fig 4.01D).

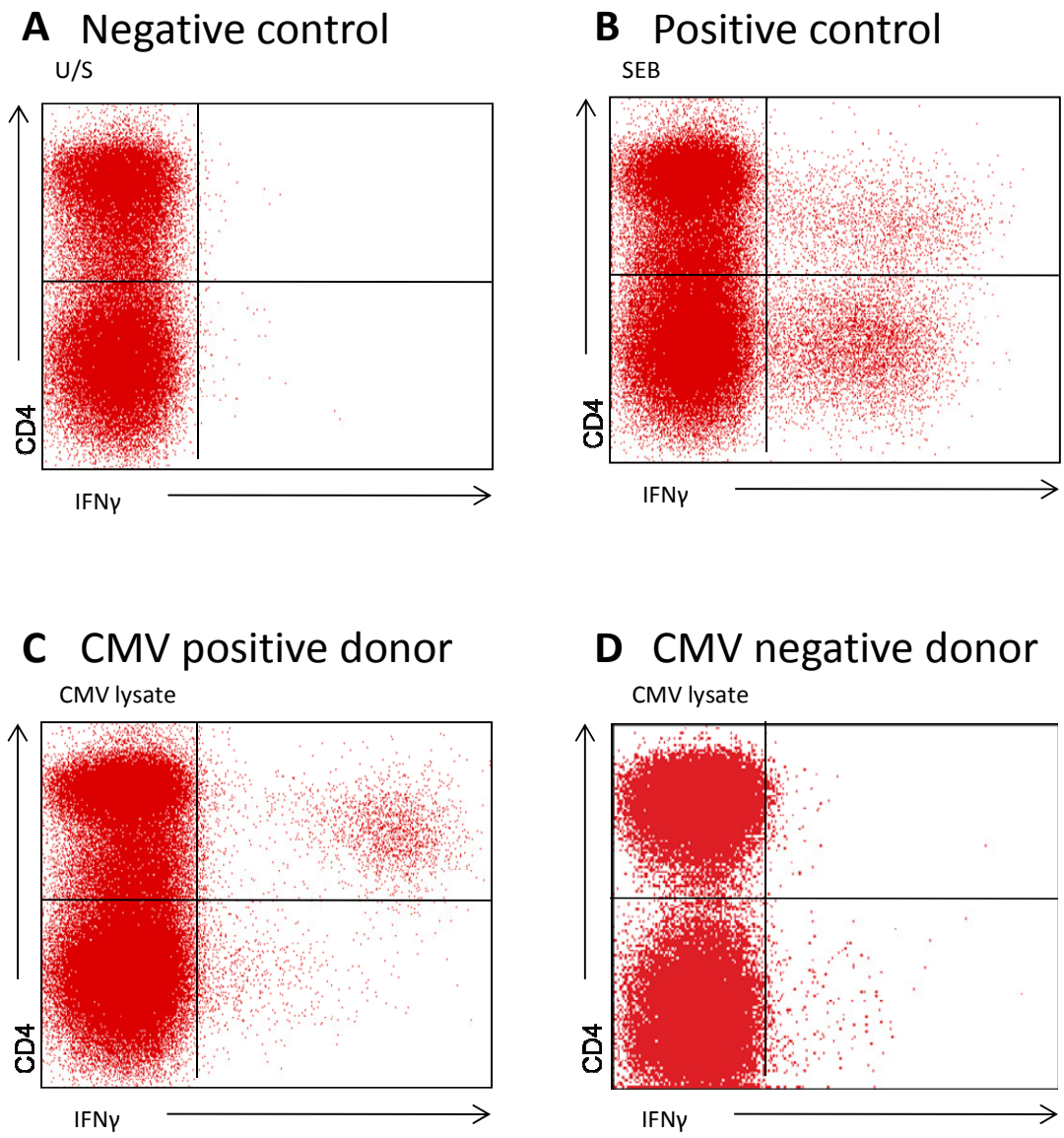


Figure 4.01 Characterisation of donors CMV status.

Purified PBMCs from donors were **(A)** left unstimulated, as a negative control, **(B)** stimulated with the superantigen SEB as a positive control, or **(C,D)** stimulated with CMV lysate for 18hours. Following a 6 hour Brefaldin A incubation, they were then stained with CD4 PerCP and intracellularly with IFNγ-APC. **(C) D**onors whose $CD4^+$ T cells upregulated significant amounts of intracellular IFNγ were deemed CMV positive **(D)** and those who did not were labelled CMV negative

4.2.2 Elucidation of CMV specific CD8$^+$ T cells

HLA-A2 and HLA-B7 antibodies and their isotype controls were used to stain PBMCs and determine a donor's A2/B7 status (Fig 4.02A, isotype control; Fig 4.02B, representative example of HLA-B7$^+$A2$^-$ donor). The CD8$^+$ T cell response is highly focussed on a small number of epitopes, most frequently against peptides derived from the pp65 protein (Wills et al., 1996). Therefore, we used HLA-A2 and HLA-B7 restricted pp65 specific pentamers to identify CMV specific CD8$^+$ T cells. HLA-A2$^+$ or -B7$^+$ donors were then stained with NLV or TPR pentamers, respectively, plus an irrelevant control pentamer, to determine if a suitable CMV specific CD8$^+$ T cell population was present (Fig 4.02C, irrelevant pentamer; Fig 4.02D, representative TPR staining of a TPR$^+$ donor).

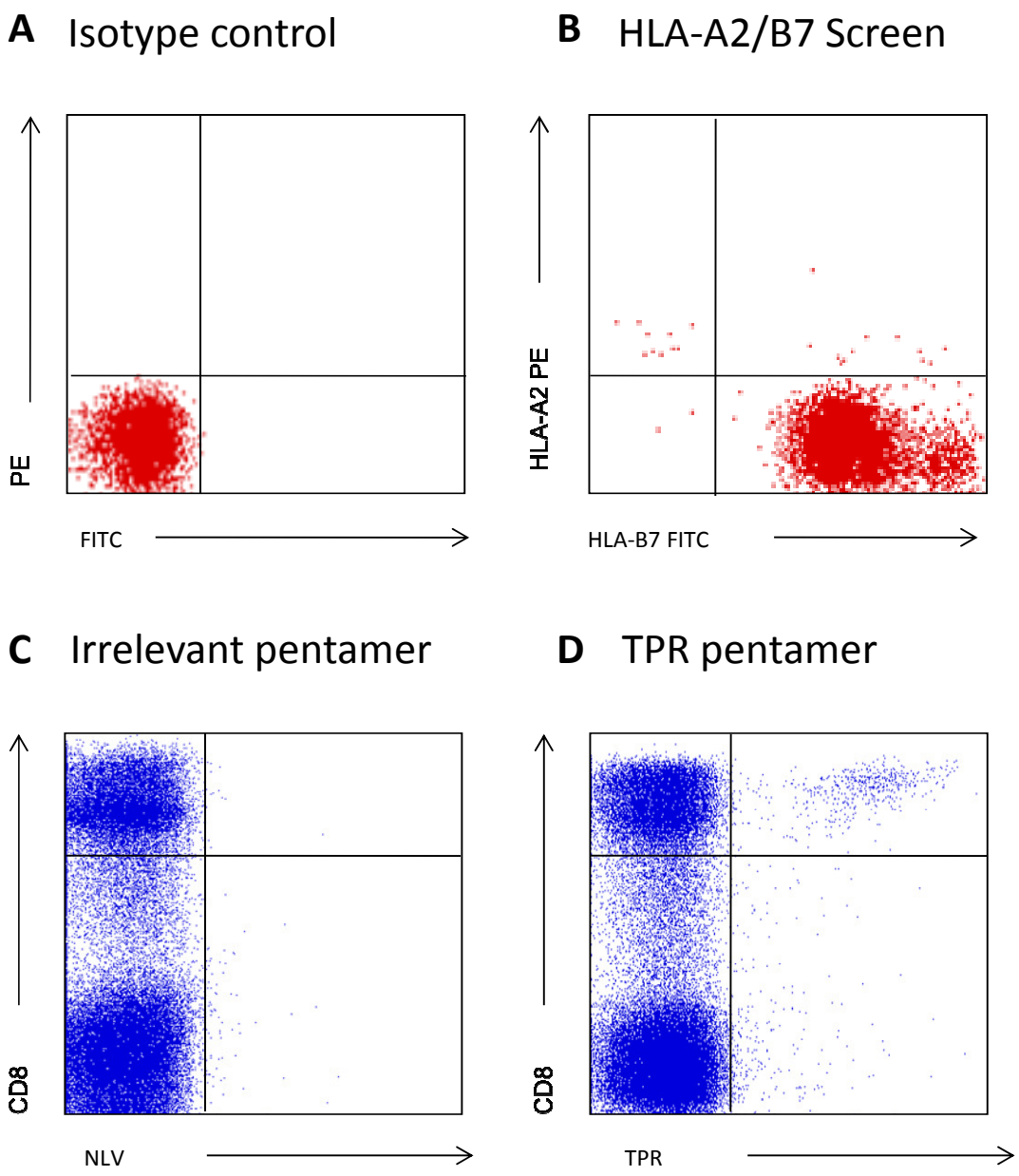


Figure 4.02 Identification of donors with pentamer positive cell populations.

Purified PBMCs were stained with HLA-A2-PE, HLA-B7-FITC or the relevant isotypes and CD8-PerCP. **(A)** Gated on CD8+ T cells, isotype control staining and **(B)** HLA- A2 and -B7 staining of a HLA-A2^{-}B7^{+} donor is displayed. HLA-B7^{+} or HLA-A2^{+}B7^{-} donors were then stained with TPR or NLV pentamer, respectively to identify CMV specific CD8^{+} T cells. **(C)** Gated on live lymphocytes, a HLA-B7^{+}TPR^{+} donor PBMCs stained with an irrelevant peptide-pentamer (NLV) or **(D)** TPR peptide-pentamer.

4.2.3 Phenotypic definition of pp65CMV specific CD8$^+$ T cells

Utilising the CD27/CD45RA phenotypic markers (as described in Fig 3.01) it was determined that CMV specific CD8$^+$ T cells are significantly more differentiated than the pentamer negative CD8$^+$ pool from the same young donors containing significantly fewer naïve cells alongside a concomitant increase in effector memory cells (Fig 4.03A, representative example; Fig 4.03B, grouped data) and also from a single representative old donor (Fig 4.03C). Moreover, the frequency of these pp65 epitope specific CD8$^+$ T cells increased with ageing (data not shown; $r^2 = 0.55$, $P < 0.01$).

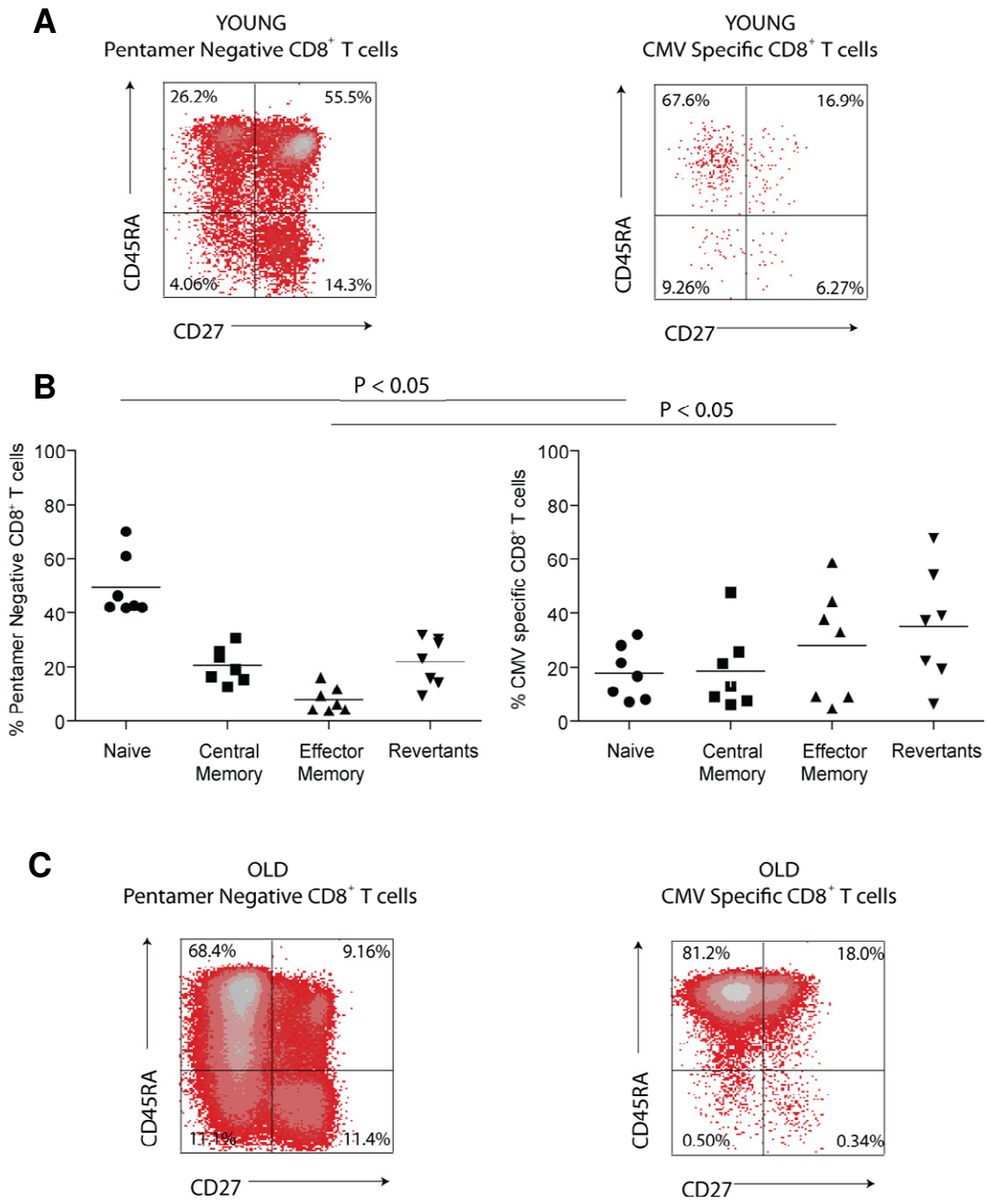


Figure 4.03 Characterisation of CMV specific CD8+ T cell phenotype.

(A). A representative example of a young donor whose PBMCs had been stained with anti-CD8-PerCP, anti-CD27-FITC, anti-CD45RA-APC and the relevant HLA-A2 or HLA-B7 restricted CMV specific pentamer. The CD8+ cells were gated on (left panel) pentamer- or (right panel) pentamer+ (for CMV specific) cells and their phenotype assessed using CD27 and CD45RA markers with the percentage of cells present in each quadrant displayed. **(B)** Pooled data showing CD45RA and CD27 expression on the pentamer negative CD8+ pool and on virus specific CD8+ T cells on 7 young donors. Horizontal lines depict mean values. P values calculated using a Wilcoxon signed rank test, only significant differences between CMV specific CD8s and their pentamer negative counterparts indicated. **(C)** Representative dot plots showing CD45RA and CD27 expression on the CMV specific and pentamer negative CD8+ T cells from an old individual (aged 91).

4.2.4 Inhibitory receptor expression on CMV specific CD8⁺ T cells

Inhibitory receptor expression was characterised on CMV specific $CD8^+$ T cells, identified as described above. CTLA-4 expression was observed at low levels on CMV specific cells in both young and old individuals; approximately half of all CMV specific cells expressed PD-1; KLRG1 expression was observed at high levels on CMV specific cells and was further upregulated on CMV specific cells of old versus young donors (Fig 4.04).

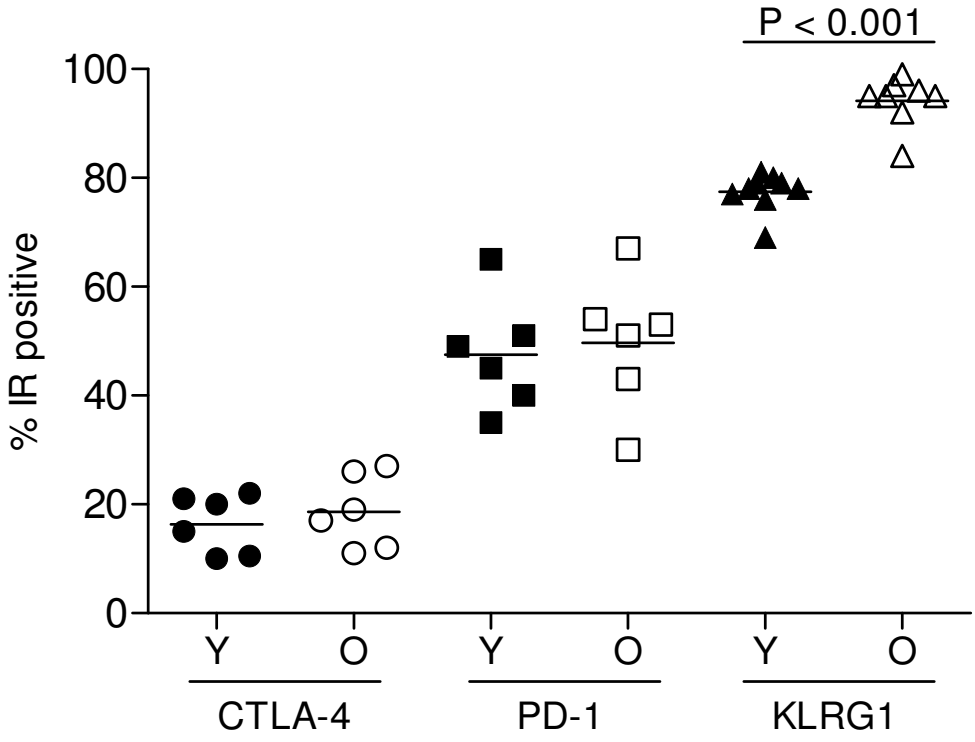


Figure 4.04. Comparison of CTLA-4, PD-1 and KLRG1 levels on CMV specific $CD8^+$ T cells in the old and young.

Peripheral blood from young and old donors was stained with APC labelled HLA-A2 and HLA-B7 restricted pentamers plus anti- CD8-PerCP and either PD-1-PE, CTLA-4-PE or KLRG1-PE antibodies. Graph illustrates the percentage of CMV specific cells from young, Y, <35 years, and old, O, >65, donors, that express CTLA-4, PD-1 and KLRG1. Horizontal bars depict mean vales. Statistical differences calculated using a Man-Whitney U test and only significant differences between young and old donors for a given inhibitor receptor are displayed.

4.3 Determining the contribution of inhibitory receptor signalling to CMV specific $CD8^+$ T cell proliferative dysfunction

The contribution of inhibitory receptor expression towards the dysfunction of CMV specific $CD8^+$ T cells was investigated using antibody blocking studies. This first required the identification of CMV^+ donors who possessed the HLA alleles B7 or A2 together with a sufficiently large population of CMV specific $CD8^+$ T cells (Fig 4.05). The proliferative responses of CMV specific $CD8^+$ T cells were determined using the proliferative marker Ki67, a nuclear antigen strictly associated with cellular proliferation (Scholzen and Gerdes, 2000). Ki67 was expressed at low levels directly e*x vivo* by bulk $CD8^+$& $CD4^+$ T cells (Fig 4.06) and CMV specific $CD8^+$ T cells (Fig 4.07). Moreover, Ki67 was expressed at low levels throughout all $CD8^+$ and $CD4^+$ T cell differentiation stages, being most highly expressed on central and effector memory populations of $CD8^+$ T cells and reaching maximal levels on effector memory $CD4^+$ T cells (Fig 4.08)

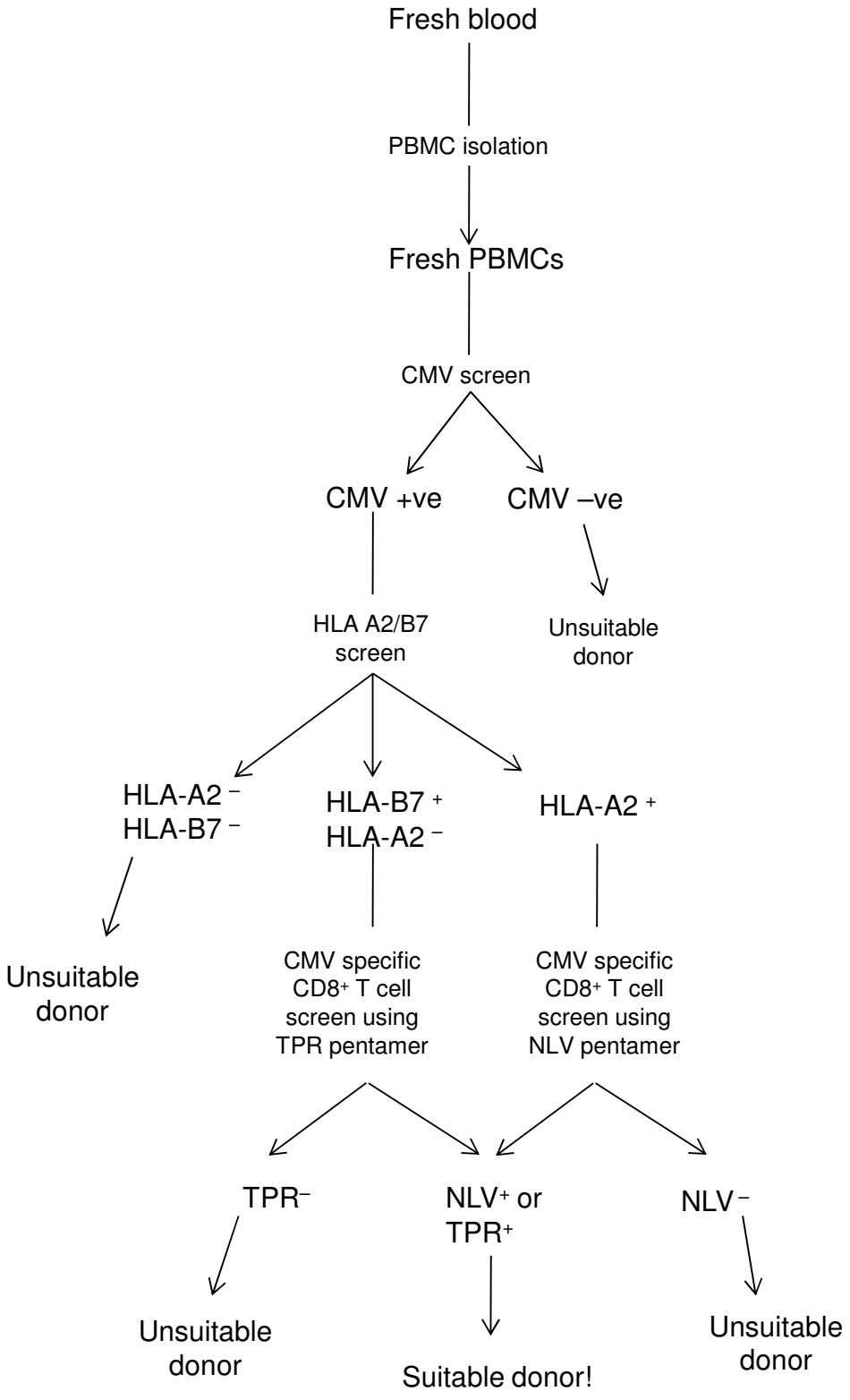


Figure 4.05 Schematic for identification of suitable donors for the experiment to investigate the effects of inhibitory receptor blockade on CMV specific CD8+ T cells.

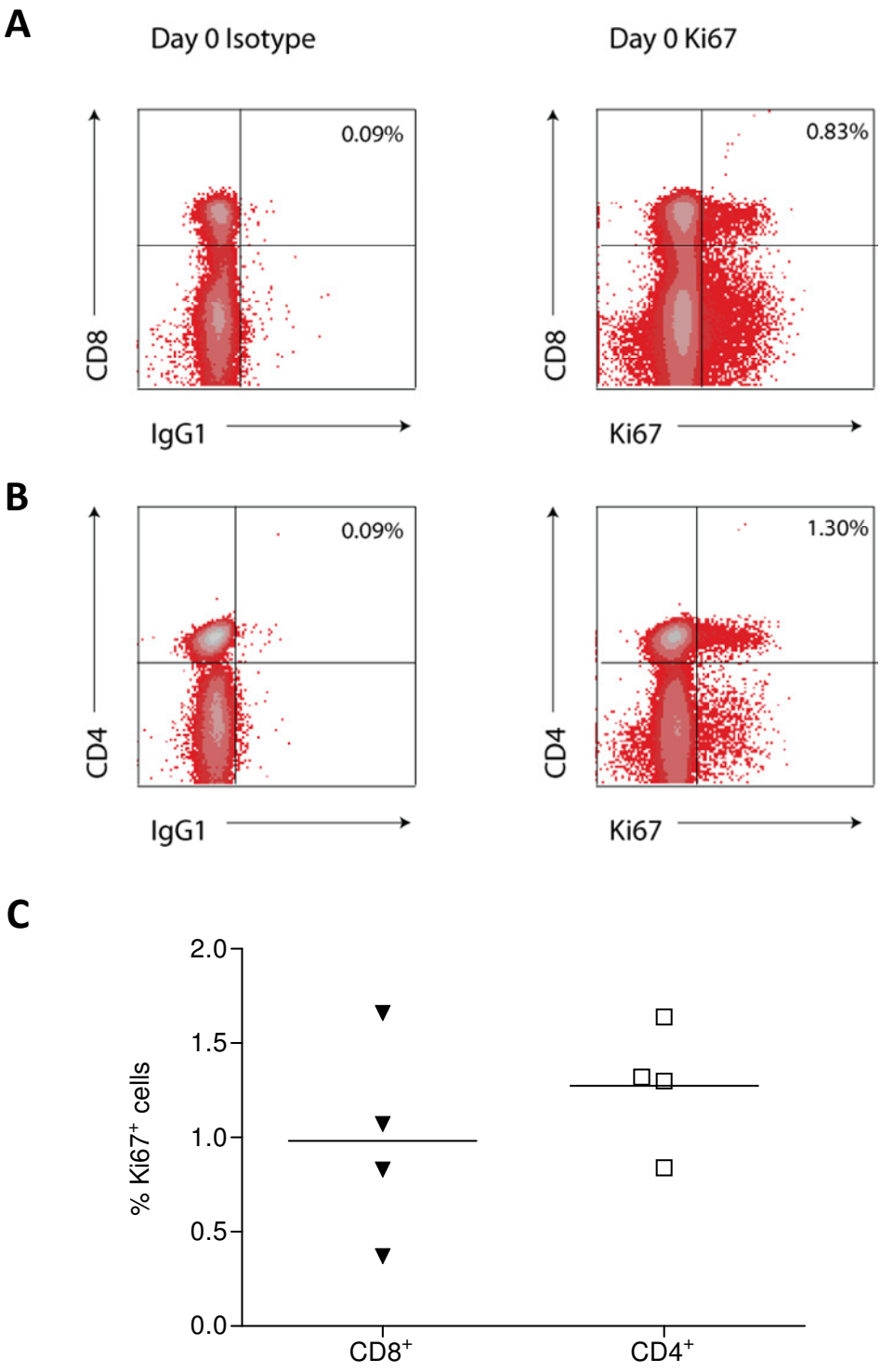


Figure 4.06. ***Ex vivo*** **Ki67 expression on CD8⁺ and CD4⁺ T cells.**

Purified fresh PBMCs were stained with anti- CD8-PerCP or CD4-PerCP and intranuclearly with KI67-FITC. **(A)** Representative density dot plots gated on live lymphocyte populations illustrating $CD8^+$ T cells and **(B)** $CD4^+$ T cells from the same donor and their expression of (right panels) Ki67 or (left panels) its isotype control antibody. **(C)** Pooled data from 4 donors illustrating the proportion of $CD8^+$ and $CD4^+$ T cells that stained positively for Ki67 expression. Horizontal bars indicate mean values. A Mann-Whitney U test was used to generate P values but no significant difference was generated.

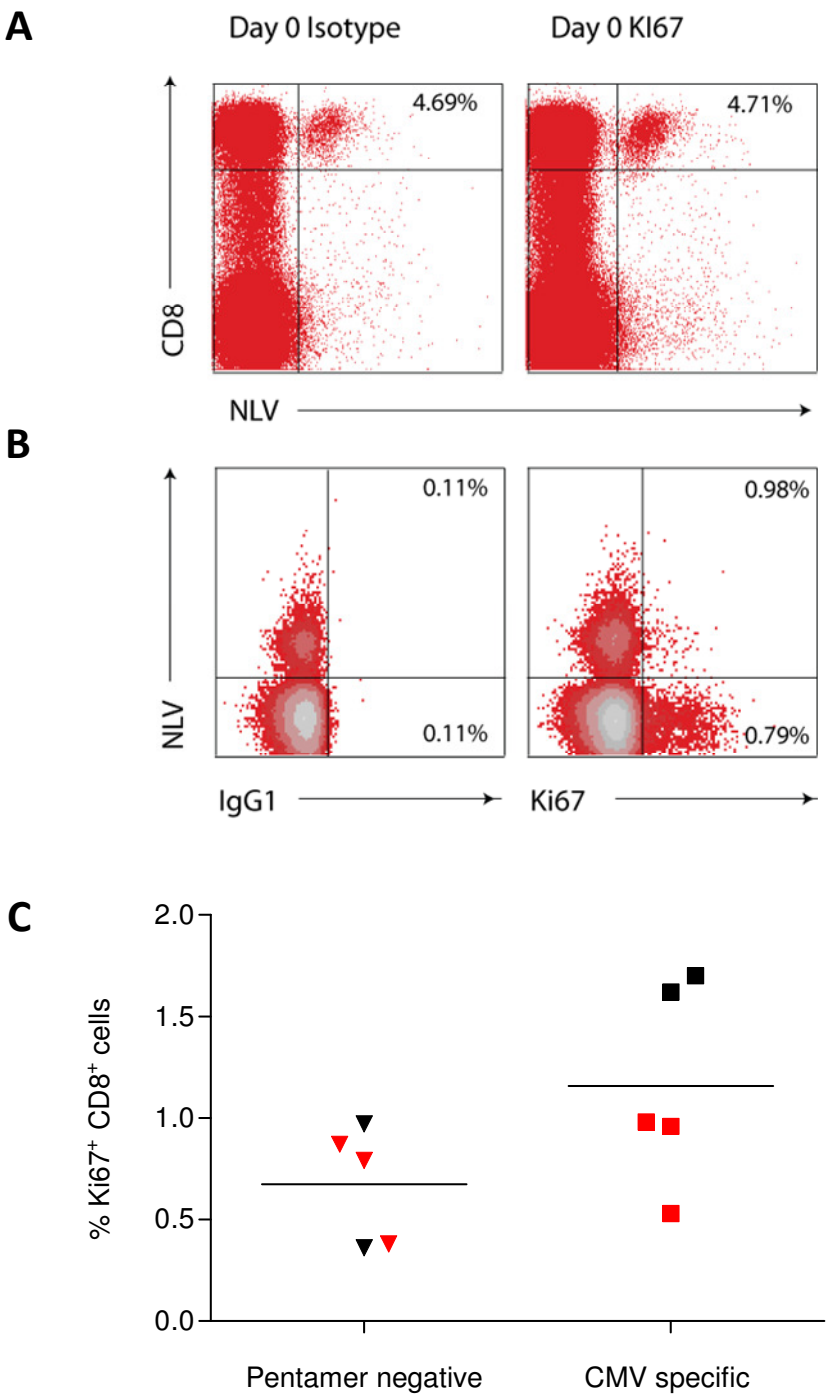


Figure 4.07. ***Ex vivo*** **Ki67 expression on CMV specific CD8⁺ T cells.**

Purified fresh peripheral blood cells were stained with anti- CD8-PerCP and NLV or TPR specific PE labelled pentamers plus anti-Ki67-FITC intranuclearly **(A)** Representative FACS plots gated on live lymphocytes illustrating the identification of CMV specific CD8⁺ T cells and **(B)** their expression of (right panels) Ki67 or (left panels) its isotype control with the proportion of CMV specific and pentamer negative cells expressing Ki67 indicated. **(C)** Pooled data illustrating the proportion of CMV specific and pentamer negative CD8⁺ T cells that stained positively for Ki67 expression. The black dots represent TPR⁺ donors and the red dots indicate NLV⁺ donors. Horizontal bars indicate mean values. P values generated using a Mann-Whitney U test. Only significant differences are displayed.

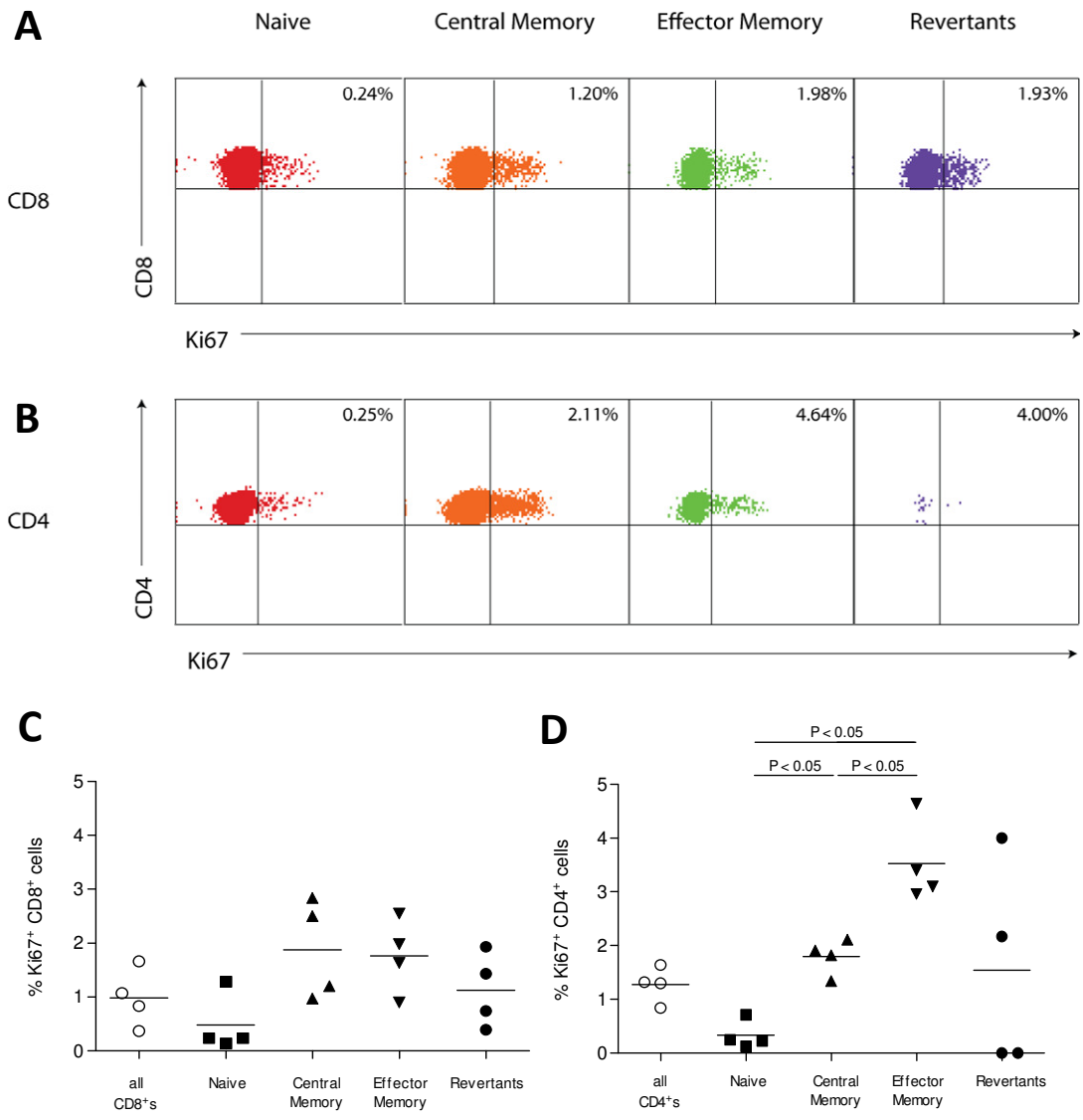


Figure 4.08. Ki67 expression on CD8⁺ and CD4⁺ T cells *ex vivo* vary with stage of differentiation.

Freshly isolated PBMCs were stained with anti- CD8-PerCP or CD4-PerCP, CD27-PE, CD45RA and intranuclearly with anti-Ki67-FITC and analysed by flow cytometry. **(A)** Representative dot plots illustrating the proportion of CD8⁺ and **(B)** CD4⁺ T cell subsets, identified using CD27 and CD45RA markers, as described earlier, that express Ki67. **(C)** Pooled data of 4 donors illustrating the proportion of CD8⁺ and **(D)** CD4⁺ T cells and their subsets that stained positively for Ki67 expression. Horizontal bars illustrate mean values. P values calculated using a Mann Whitney U test. Significant differences in Ki67 expression between different subsets are indicated.

4.3.1 PD-1/L blockade reverses the proliferative defect of CMV specific CD8$^+$ T cells

Fresh PBMCs, from donors with a suitable population of CMV specific CD8$^+$ T cells, were stimulated for 3 days with CMV peptide, and the effect of inhibitory receptor blockade on the size of the CMV response and their proliferative capacity was determined, as measured by the expression of Ki67 (Fig 4.09). The pp65CMV peptide was titrated to find the concentration that would be sufficiently high to induce an adequate proliferative response, as measured by Ki67 expression of pentamer$^+$ CD8$^+$ T cells, (Fig 4.10C), but not be so high that it would be toxic to the pentamer$^+$ CD8$^+$ T cells, as determined by the size of the pentamer response (Fig 4.10B). Thus, 0.2µg/ml was determined appropriate. Of the inhibitory blocks tested, only PD-L1/2 blockade produced significant increases in the proliferative responses of CMV specific CD8$^+$ T cells, compared to its relevant isotype control (Fig 4.11), which was similarly observed in young and old donors (Fig 4.12A,B). This increased proliferative response was accompanied by a significant increase in the size of the CMV specific CD8$^+$ T cell response (Fig 4.13). The simultaneous blockade of PD-L, CTLA-4 and E-cadherin failed to show any synergistic or additional effects on either the proliferation (Fig 4.14) or the magnitude (data not shown) of CMV specific CD8$^+$ T cell response of a young and an old donor. Moreover, in the absence of any pp65CMV peptide stimulus, blocking PD-L for 3 days did not upregulate the size (Fig 4.15, left panels) or proliferation (Fig 4.15, right panels) of the CMV specific CD8$^+$ T cell response.

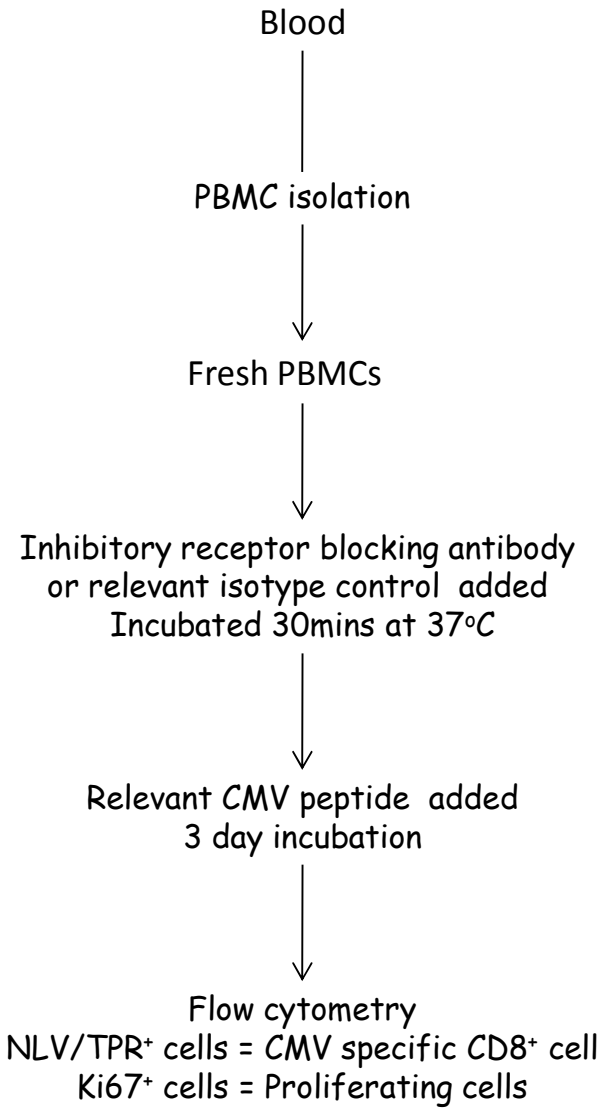


Figure 4.09 Schematic for the experiment to determine the effects of inhibitory receptor blockade on CMV specific $CD8^+$ T cell size and function

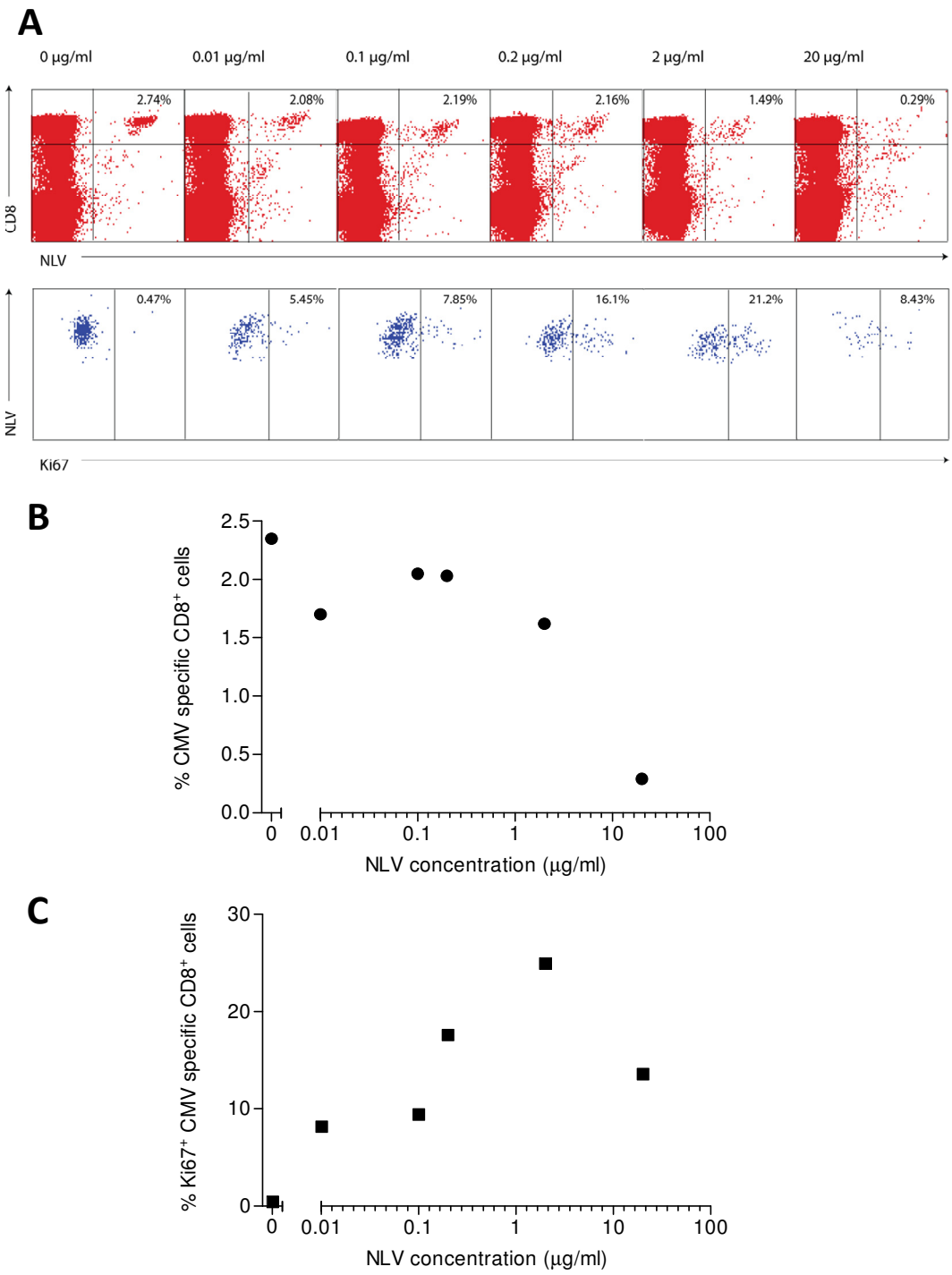


Figure 4.10 pp65 CMV peptide titration for optimal CMV specific CD8⁺ T cell proliferation.

Freshly isolated PBMCs were stimulated for 3 days with different concentrations of NLV peptide and then stained with anti- CD8-PerCP, NLV-PE and intranuclearly with anti- Ki67-FITC and then analysed using flow cytometry. **(A)** FACS plots of the frequency of (upper panels, red dots) CMV specific CD8⁺ T cells and (lower panels, blue dots) their Ki67 expression after 3 day incubation with different concentrations of CMV peptide. **(B)** Cumulative data illustrating the effects of stimulating PBMCs with different concentration of NLV peptide on the proportion of CD8⁺ T cells that are NLV⁺ and **(C)** on the percentage of these NLV⁺ cells that express Ki67.

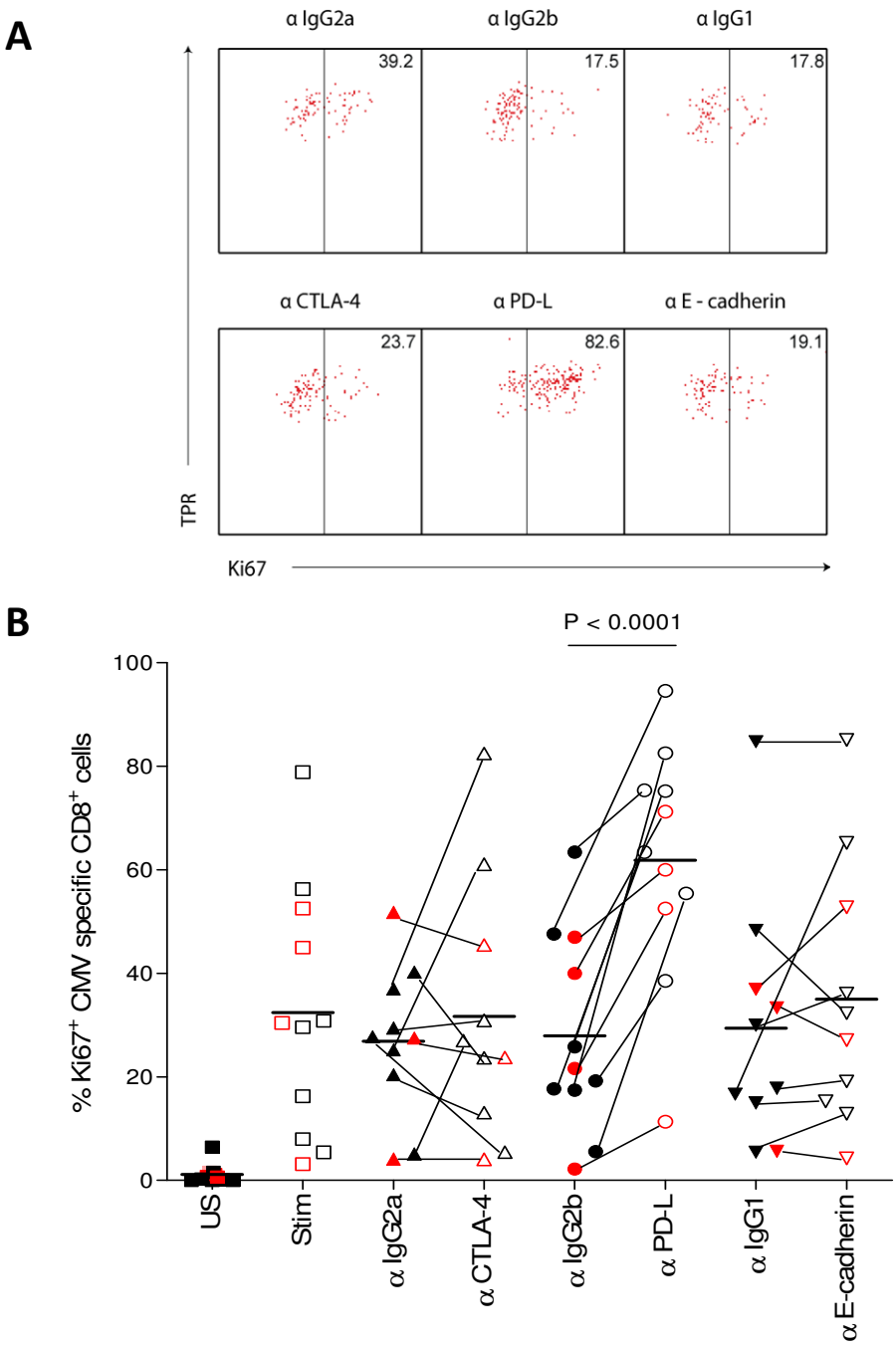


Figure 4.11 Blocking PD-1/PD-L interactions significantly enhanced CMV specific CD8⁺ T cell proliferative responses, whereas no such effect was observed by blocking CTLA-4 or KLRG1 interactions with their ligands

0.2µg/ml NLV or TPR CMV peptide was added to freshly purified PBMCs for 3 days in the presence of αCTLA-4, αPD-L1/2 or αE-cadherin blocking antibody or their relevant isotype controls, as described in Fig 4.10. The cells were then stained with CD8-PerCP, NLV or TPR-PE pentamer and intranuclearly with Ki67-FITC. **(A)** Representative FACS plots, gated on TPR⁺ or NLV⁺ CD8+ T cells, of the proportion of peptide stimulated CMV specific CD8⁺ T cells that are Ki67⁺ following blockade of different inhibitory receptors. **(B)** Pooled data of the effects of inhibitory receptor blockade, compared with their relevant isotype control antibody, on the proliferation of NLV or TPR specific cells. The red dots indicate NLV⁺ donors and the black dots represent TPR⁺ donors. Horizontal lines depict mean values. The P values were calculated using a paired Student's t-test. Significant differences between the inhibitory receptor block and their relevant isotype controls are shown.

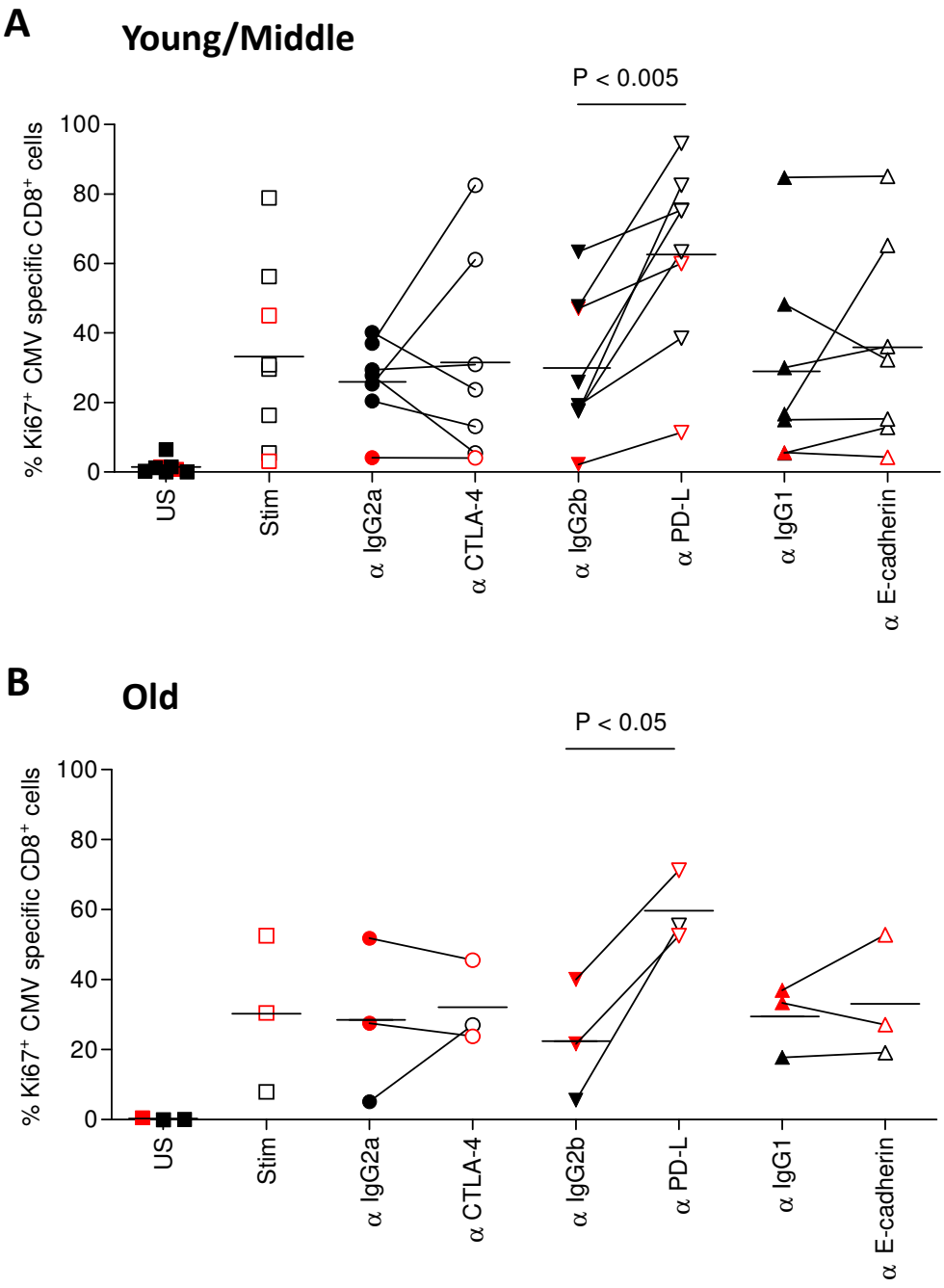


Figure 4.12 Comparing the effects of blocking inhibitory receptors on the proliferative responses of CMV specific CD8⁺ T cells in the old and young.

PBMCs from young/middle (<55 years, n =8) or old (>65 years, n= 3) individuals were incubated with 0.2µg/ml NLV or TPR peptide for 3 days in the presence of CTLA-4, PD-L or E-cadherin blocking antibody or their relevant isotype control. The cells were then stained with NLV or TPR-PE, CD8-PerCP and intranuclearly with Ki67-FITC. **(A)** Pooled data displaying the proportion of NLV and TPR specific cells expressing Ki67 from young/middle and **(B)** old donors following CMV peptide stimulation in the presence of different inhibitory receptor blockades The red dots indicate NLV⁺ donors and the black dots represent TPR⁺ donors. Horizontal lines depict mean values. The P values were calculated using Student's paired t-test and only significant differences between the inhibitory receptor block and their relevant isotype controls are shown.

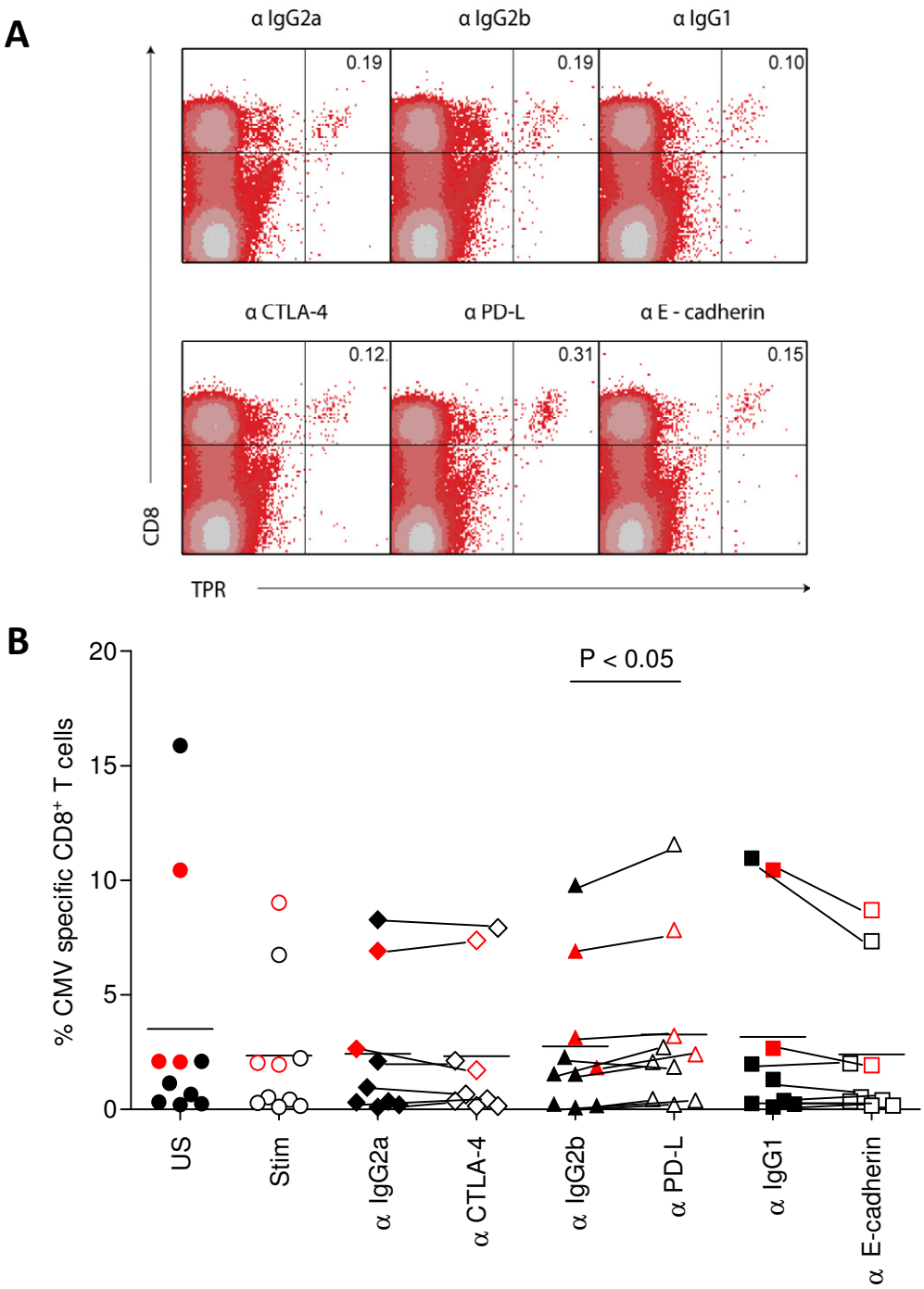


Figure 4.13 The effects of blocking inhibitory receptors on the size of the CMV specific CD8⁺ T cell response

PBMCs were stimulated and stained as described in the previous figure. **(A)** Representative FACS plots gated on live lymphocytes of changes in the proportion of CD8⁺ T cells that are NLV⁺ or TPR⁺ following 3 day CMV peptide stimulation in the presence of CTLA-4, PD-L or E-cadherin blockade and their relevant isotype controls. The percentage of CD8⁺ T cells that are TPR⁺ are indicated. **(B)** Cumulative data illustrating effects of inhibitory receptor blockade on the proportion of CD8⁺ T cells that are CMV specific. The red dots indicate NLV⁺ donors and the black dots represent TPR⁺ donors. Horizontal lines depict mean values. The P values were calculated using a Wilcoxon signed rank test. Significant differences between the inhibitory receptor block and their relevant isotype controls are shown.

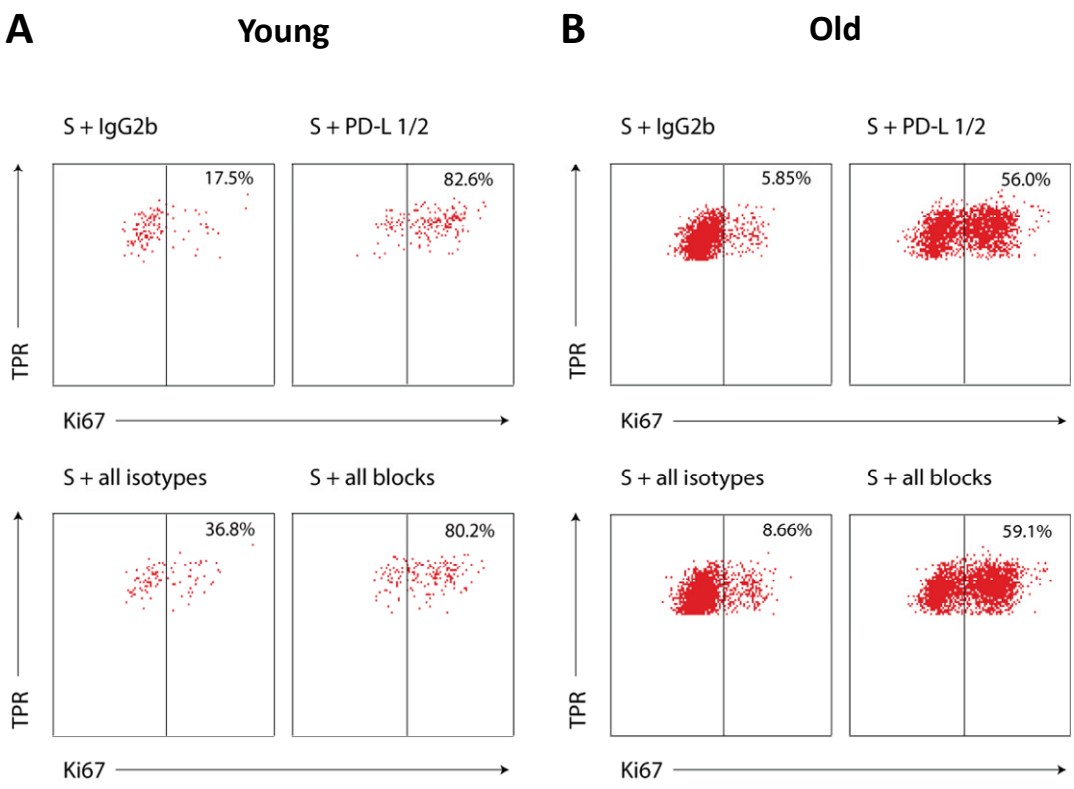


Figure 4.14 Effects of concurrently blocking PD-L, CTLA-4 and E-cadherin on CMV specific CD8⁺ T cells proliferative responses

Freshly isolated PBMCs were subjected to a 3 day TPR peptide stimulation in the presence of either PD-L, E-cadherin and CTLA-4 blocking antibodies together, PD-L blockade alone or the relevant isotype controls. The cells were then stained with TPR-PE pentamer, anti- CD8-PerCP and intranuclearly with anti-Ki67-FITC and analysed by flow cytometry. **(A)** Representative dot plots gated on TPR^+ $CD8^+$ T cells showing alterations in the percentage of CMV specific $CD8^+$ T cells expressing Ki67 following a 3 day CMV peptide stimulus in the presence of PD-L, CTLA-4 and E-cadherin together, PD-L blockade alone or the relevant isotype control, in a young and **(B)** an old individual.

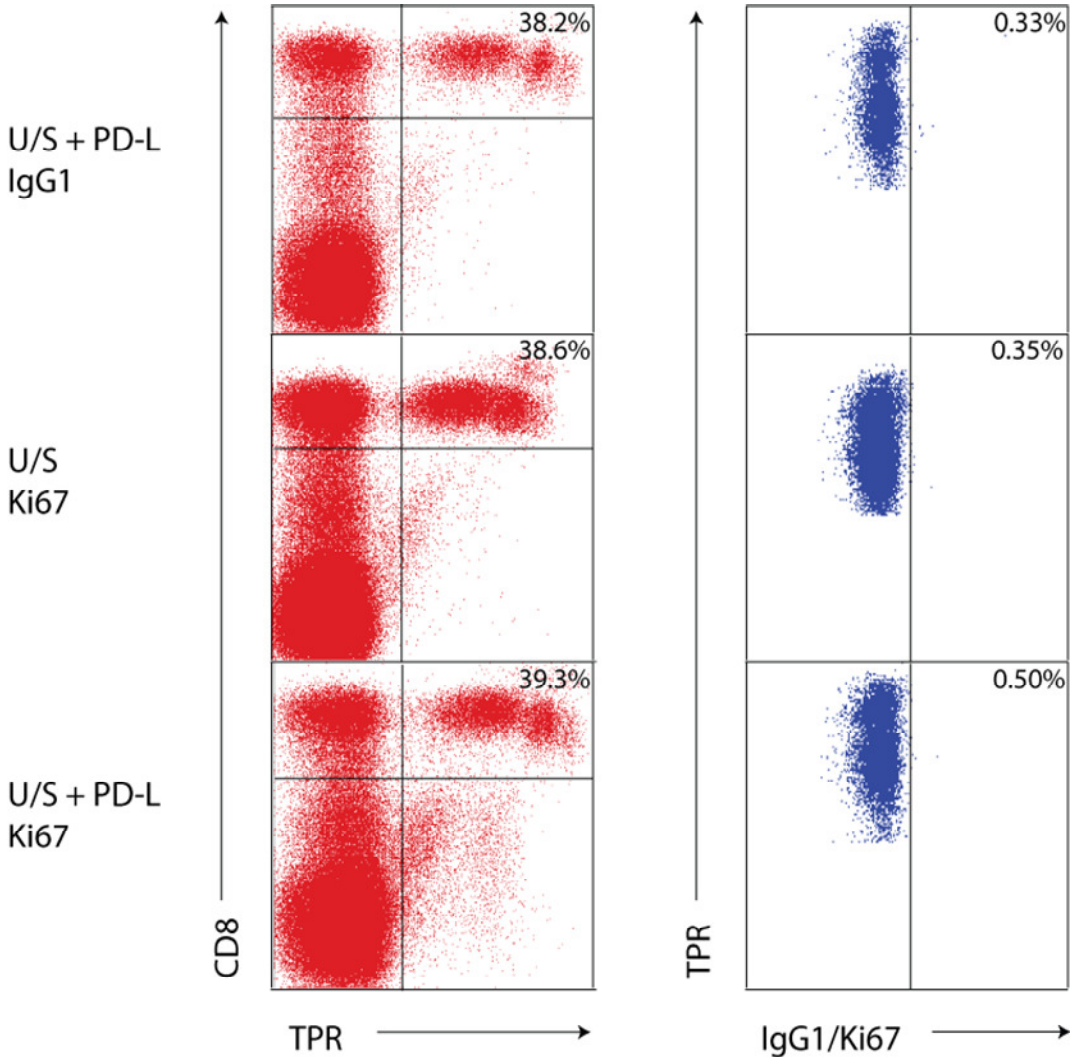


Figure 4.15 The effects of blocking PD-L on CMV specific CD8⁺ T cells in the absence of any stimulus

Fresh purified PBMCs were incubated with or without PD-L blockade for 3 days. The cells were then stained with anti-CD8-PerCP, TPR-PE and intra-nuclearly with either anti-Ki67-FITC or its isotype control-FITC and analysed by flow cytometry. (Left panels) Dot plots of changes in the proportion of CD8⁺ T cells specific for CMV and (right panels) the expression of Ki67 by these CMV specific cells either **(A)** following 3 days PD-L blockade and isotype control staining, or **(B)** Ki67 staining in the absence or **(C)** presence of PD-L blockade.

4.3.2 CMV specific CD45RA-revertant memory CD8$^+$ T cells exhibit diminished proliferative responses compared with their central and effector memory counterparts, which are largely reversed following PD-L blockade

The effects of PD-L blockade on the proliferative responses of CMV specific CD8$^+$ T cells were further explored by stratifying the results using T cell phenotypic markers. CMV specific CD45RA-revertant memory CD8$^+$ T cells were found to elicit a lesser proliferative response to pp65CMV peptide, compared with their central and effector memory counterparts (Fig 4.16). However, following PD-L blockade, the proliferative responses of CD45RA-revertant memory CMV specific CD8$^+$ T cells were augmented by a significantly greater magnitude than that of its central or effector memory counterparts (Fig 4.17). This largely compensated for the deficit observed in Fig 4.16. However, Fig.3.10 revealed that 48hrs of TCR stimulation was sufficient to induce T cell differentiation. Similarly, phenotype analysis of CMV specific CD8$^+$ T cells, following 3 day pp65 stimulation with PD-L blockade, revealed that significant phenotypic changes occurred compared to its isotype control. Indeed, reductions in the proportion of CMV specific CD8$^+$ T cells that are T_N and T_{CM} were observed, accompanied by increases in their T_{EM} and T_{REV} counterparts (data not shown).

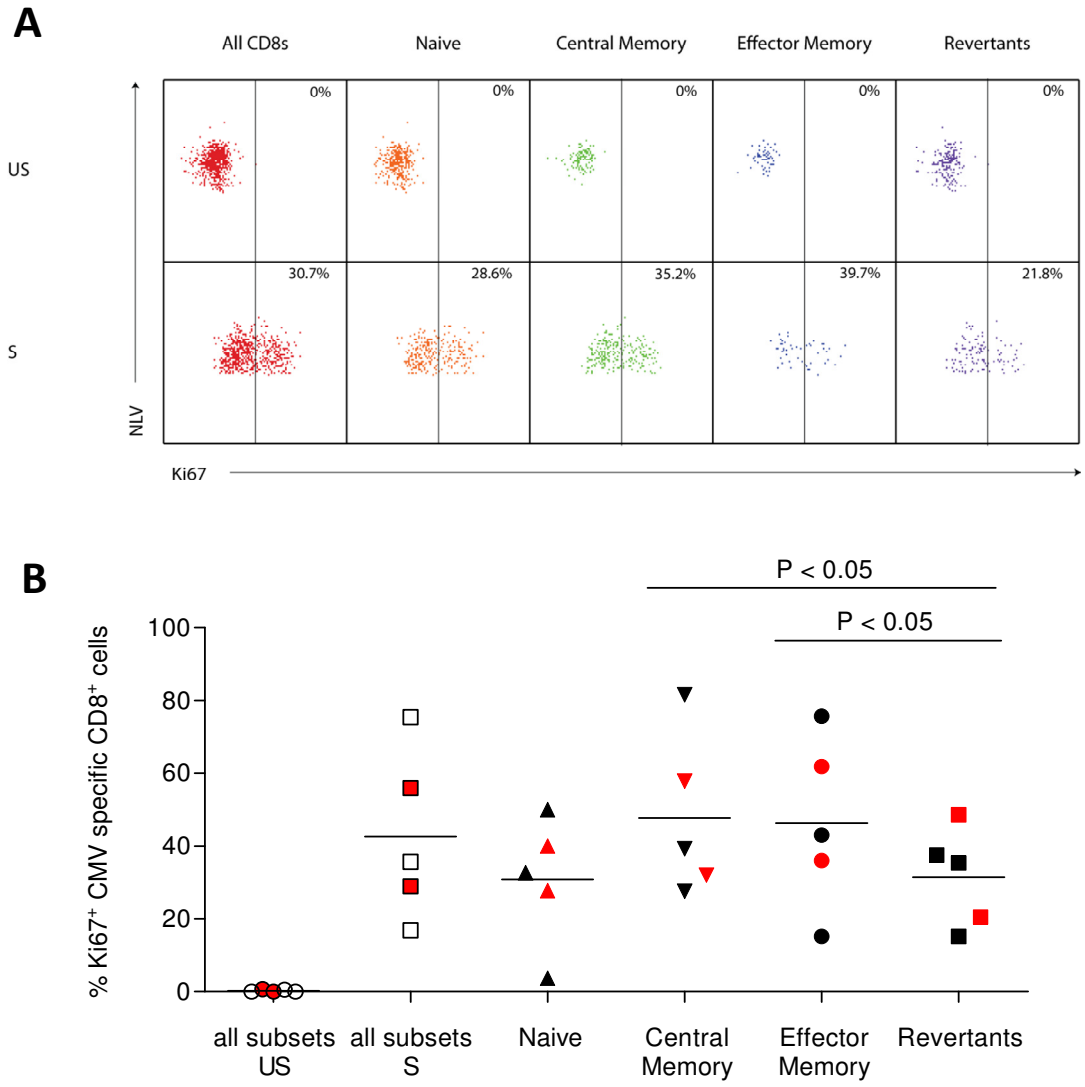


Figure 4.16 CMV specific revertant CD8⁺ cells elicit a defective proliferative response to CMV peptide compared to central and effector memory cells.

Freshly isolated PBMCs were subjected to a 3 day NLV or TPR stimulus after which they were stained with anti- CD8-PerCP, NLV or TPR-PE, CD27 APC, CD45RA-PE-Cy7 and intranuclearly with anti-Ki67-FITC. CD27/CD45RA phenotypic markers used to identify CD8⁺ T cell subsets as previously described. **(A)** Representative dot plots, gated on NLV⁺ CD8⁺ T cells, illustrating Ki67 expression on peptide stimulated CMV specific CD8⁺ T cells at different T cell differentiation stages. **(B)** Pooled data illustrating the effects of a 3 day CMV peptide stimulus on the Ki67 expression of whole CD8⁺ T cells and CD8⁺ T cell subsets. Horizontal bars depict mean values. Red dots represent NLV⁺ donors and black dots indicate TPR⁺ donors. P values assessed using a one tailed Wilcoxon signed rank test. Significant differences between revertant cells and other subsets are displayed.

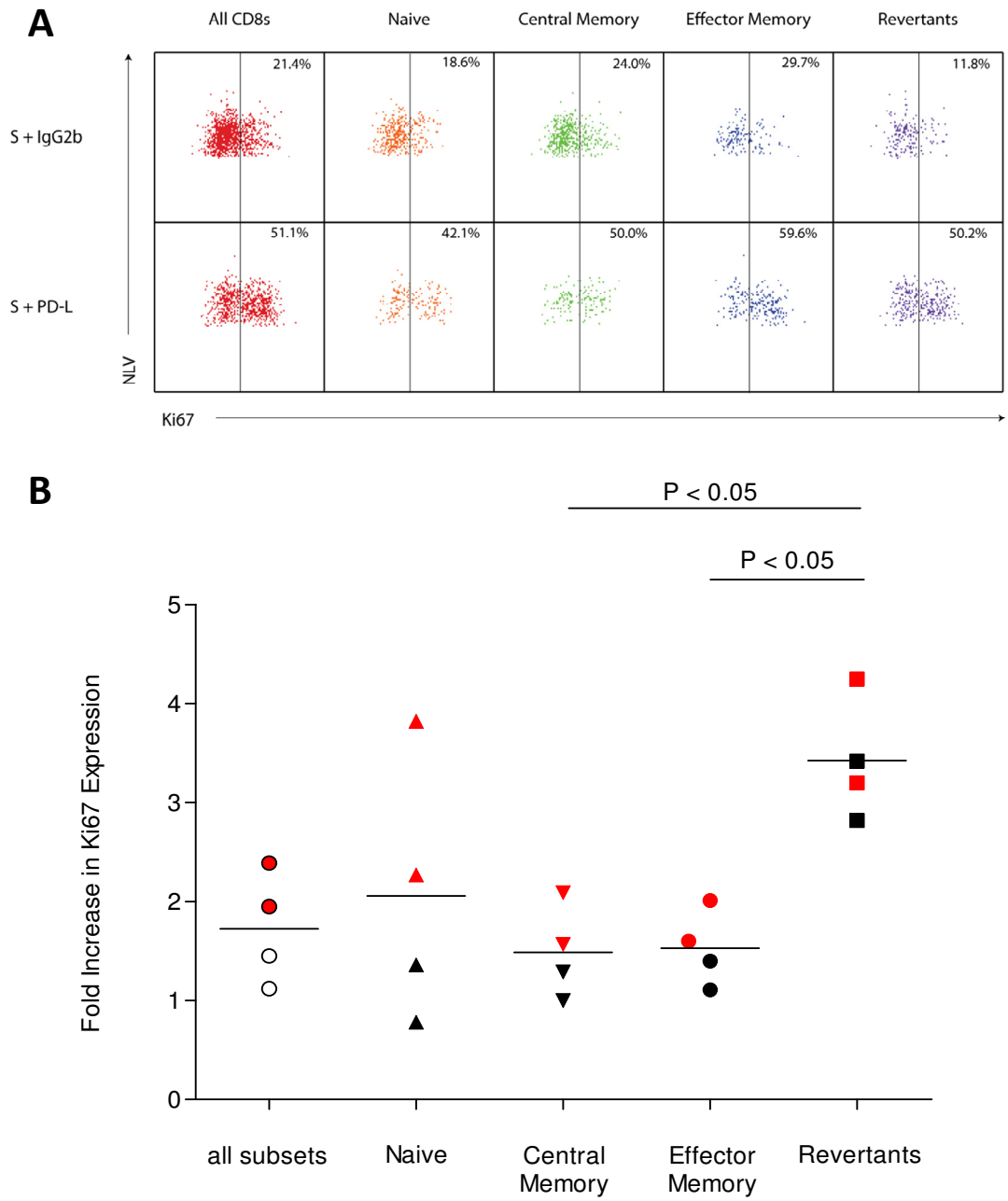


Figure 4.17. Blocking PD-1/PD-L interactions augments the proliferative responses of revertant CMV specific CD8⁺ T cell's by a greater magnitude than that of central or effector memory subsets.

PBMCs were stimulated with 0.2µg/ml TPR or NLV peptide for 3 days in the presence of PD-L block or IgG2b isotype control. Cells were then stained with anti- CD8-PerCP, TPR or NLV-PE, CD27 APC, CD45RA-PE-Cy7 and intranuclearly with Ki67-FITC. CD27 and CD45RA phenotypic markers used to define CD8 subsets as previously described. **(A)** Representative FACS plots gated on NLV⁺ CD8⁺ T cells of Ki67 staining on peptide stimulated CMV specific CD8⁺ T cell subsets in the presence of PD-L blockade compared to IgG2b isotype control. **(B)** Cumulative data illustrating effects of PD-L receptor blockade on the fold increase of the proportion of CMV specific CD8⁺ T cells subsets expressing Ki67. Red dots indicate NLV⁺ donors and black dots represent TPR⁺ donors. Horizontal bars depict mean values. A Mann-Whitney U test was used to calculate P values. Significant differences between revertant, central and effector memory subsets are indicated.

To confirm that the increased proliferative responses of CMV specific $CD8^+$ CD45RA-revertant memory T cells following PD-L blockade reflects a true reversal of their proliferative deficit, rather than central and effector memory $CD8^+$ T cells differentiating to CD45RA-revertant memory $CD8^+$ T cells, the effects of PD-L blockade on CMV specific $CD8^+$ T cells was repeated using purified CD27/CD45RA subsets. This enabled analysis of the effects of PD-L blockade on the proliferative responses of CD45RA-revertant memory $CD8^+$ T cells independent of differentiation (Fig 4.18). The purity of $CD8^+$ T cells at different stages of differentiation, obtained using FACS Aria cell separation, is depicted in Fig 4.19. This experiment revealed that, independent of differentiation, the proliferative responses of CD45RA-revertant memory $CD8^+$ CMV specific T cells undergo the strongest upregulation following PD-L blockade, compared with its isotype control (Fig 4.20), accompanied by the greatest increase in the CMV response size (Fig 4.21).

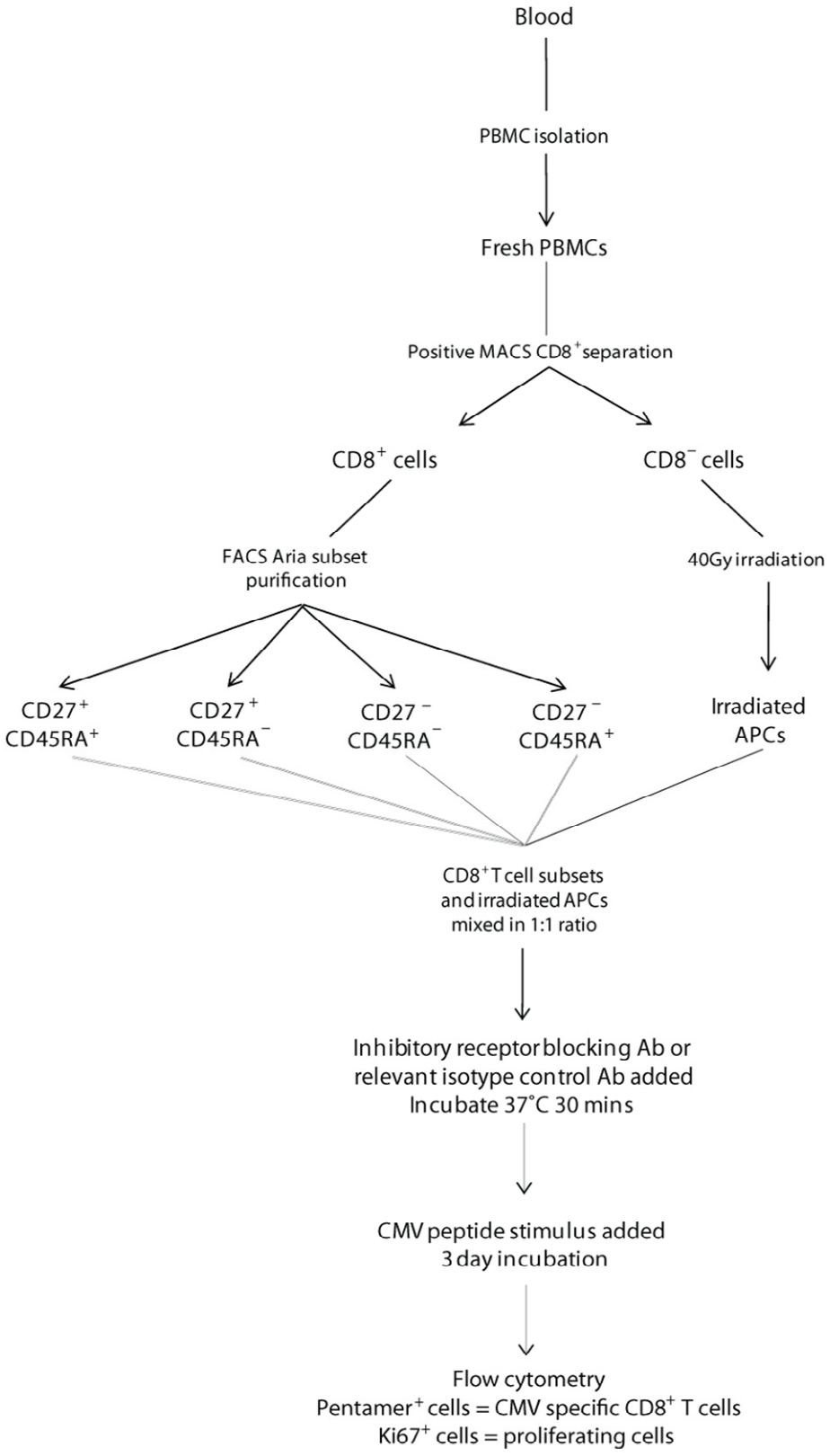


Figure 4.18 Schematic for measuring the effects of PD-L blockade on purified CD27/CD45RA subsets in response to a CMV specific stimulus

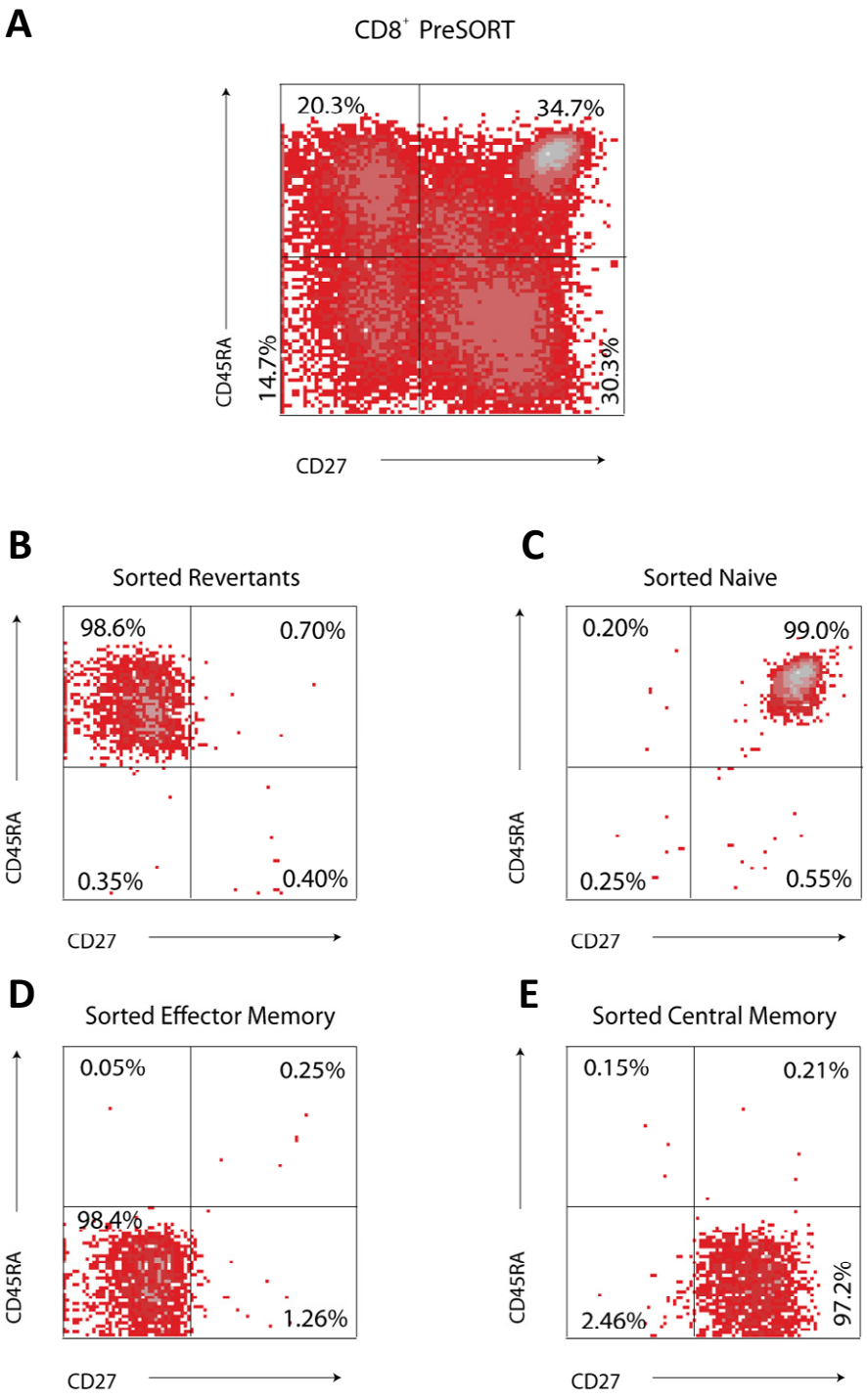


Figure 4.19 Purity of cells obtained from FACS Aria CD8⁺ T cell subset separation using CD27/CD45RA markers.

CD8+ cells were isolated from peripheral blood cells using MACS separation. CD8⁺ cells were stained with anti-CD27-PE and CD45RA-APC and then purified into 4 subsets using FACS Aria. **(A)** Representative FACS plot of CD27 and CD45RA staining on CD8⁺ T cells before sort. **(B)** Representative FACS plots showing the purity of $CD27^{-}CD45RA^{+}$, **(C)** $CD27^{+}CD45RA^{+}$, **(D)** $CD27^{-}CD45RA^{-}$ and **(E)** $CD27^{+}CD45RA^{+}$ $CD8^{+}$ T cell subsets obtained after sort with the percentage of cells occupying each quadrant indicated.

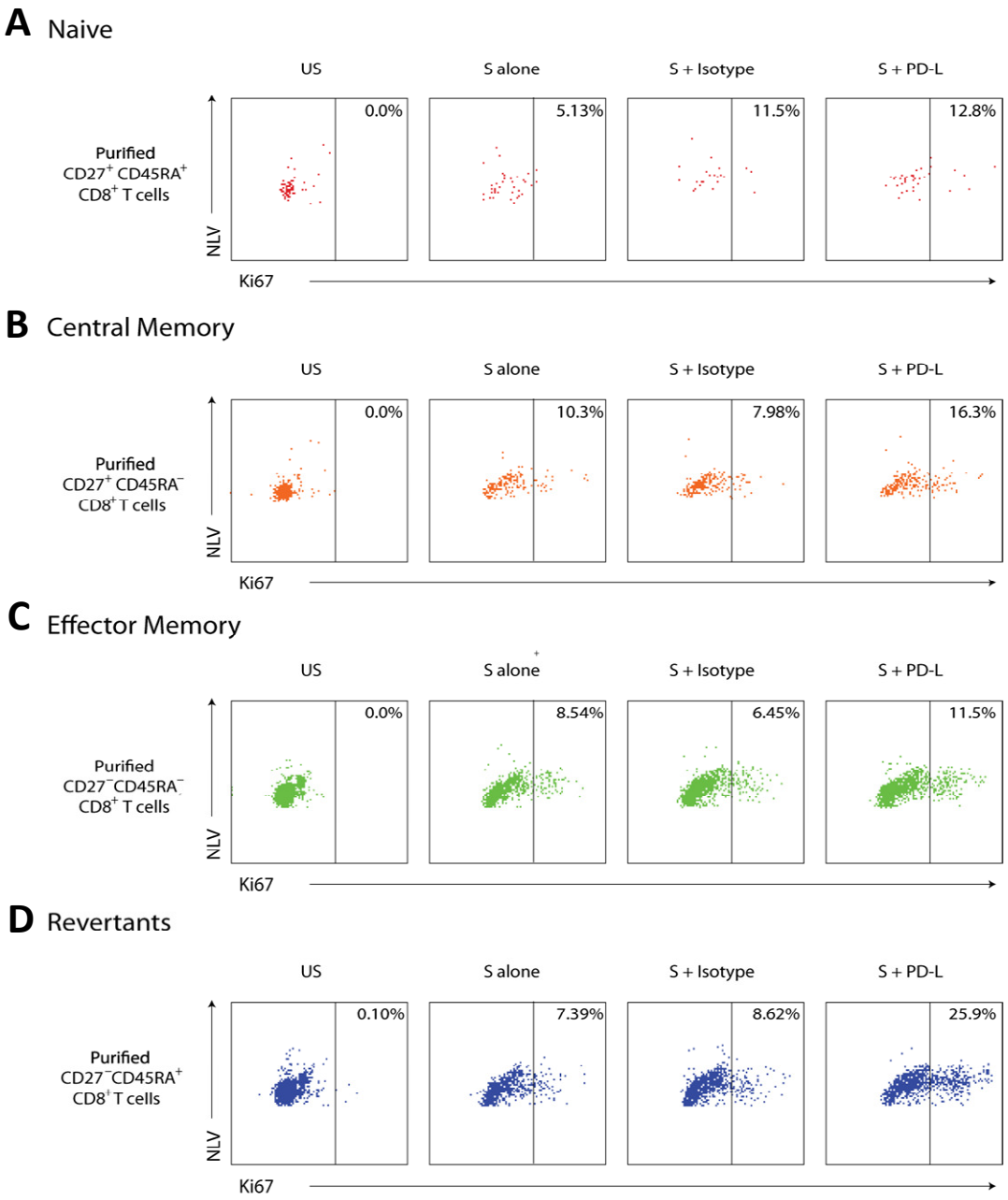


Figure 4.20 CMV specific proliferative responses in purified revertant cells undergo the strongest upregulation after blocking PD-1/PD-L interactions

CD8⁺ T cells subsets from a single donor, were purified, as described in the previous figure, and stimulated with 0.2µg/ml NLV plus irradiated APCs for 3 days in the presence of PD-L blockade or its isotype control. Cells were then stained with anti- CD8-PerCP, NLV-PE and intranuclearly with Ki67-FITC. **(A)** FACS plots of Ki67 staining on peptide stimulated CMV specific CD8⁺ T cell from purified naive, **(B)** central memory, **(C)** effector memory and **(D)** revertant subsets in the presence of PD-L blockade or their relevant isotype control. The proportion of NLV⁺ CD8⁺ T cells that express Ki67 is indicated on each plot.

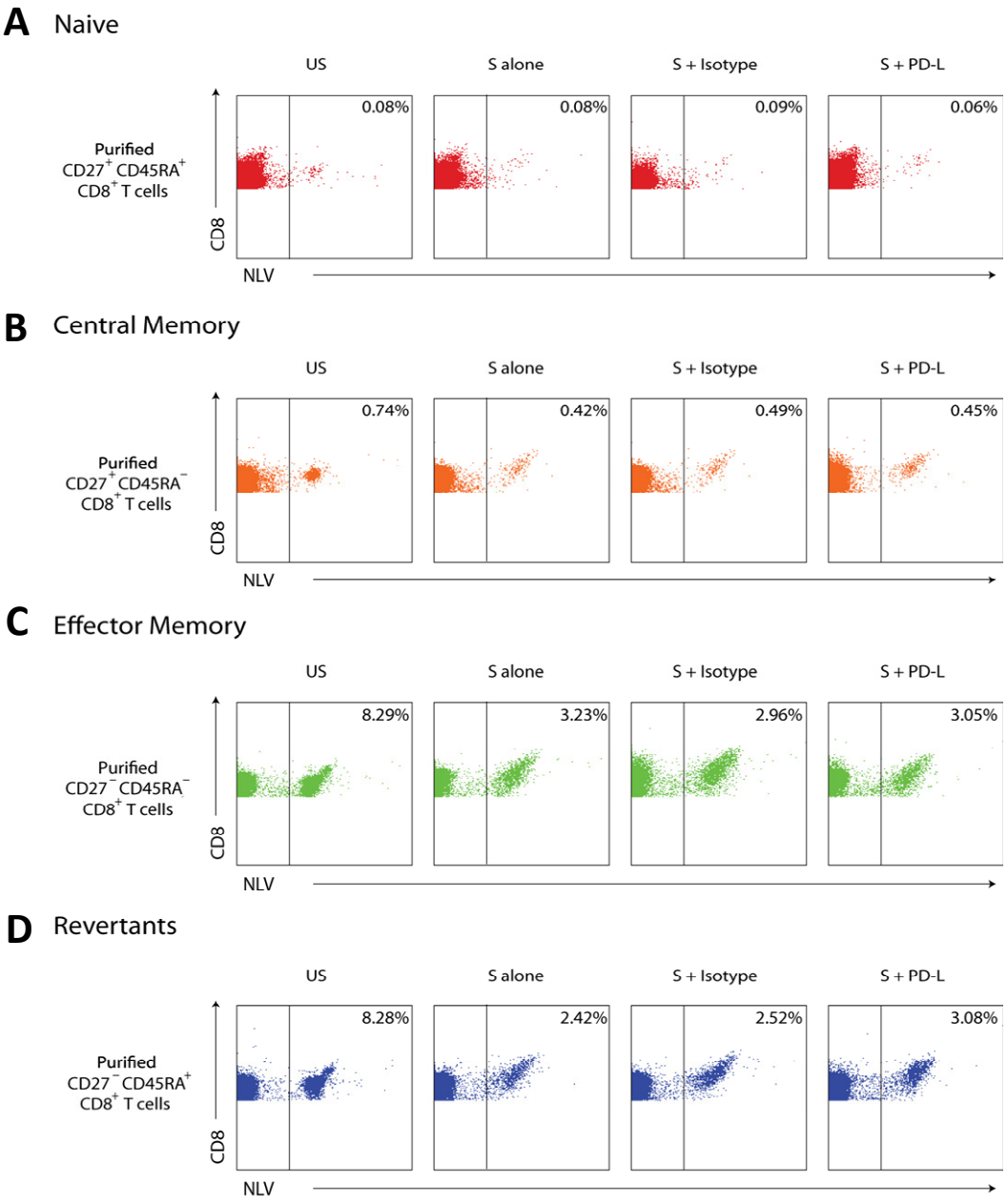


Figure 4.21 The effects of blocking PD-L on the size of the CMV specific response, from purified CD8⁺ T cell subsets

Fresh PBMCs were stimulated and stained as described in the previous figure **(A)** FACS plots of NLV staining on peptide stimulated CMV specific CD8⁺ T cell from purified naive, **(B)** central memory, **(C)** effector memory and **(D)** revertant subsets in the presence of PD-L blockade or its relevant isotype control. The percentage of CD8⁺ T cells that are NLV⁺ is indicated on each plot.

4.4 Inhibitory receptor blockade is insufficient to augment the proliferative responses of CMV specific $CD4^+$ T cells

CMV specific $CD4^+$ T cells have been documented as having a restricted replicative capacity, which becomes increasingly severe with age (Fletcher et al., 2005). We therefore examined whether inhibitory receptor blockade could reinvigorate the $CD4^+$ CMV specific T cell proliferative response. PBMCs were stimulated with CMV lysate for 3 days, which were restimulated after 48 hrs with Brefeldin added over the last 4 hrs (Fig 4.22), after which time the CMV^+ $CD4^+$ T cells were identified as those $CD4^+$ T cells being $IFN\gamma^+$, and their proliferative responses measured using Ki67 staining (Fig 4.23). The effect of inhibitory receptor blockade compared with their relevant isotype controls on the size and proliferative capacity of the $CD4^+$ CMV specific T cell response was determined. None of the inhibitory receptor blockades were able to significantly alter either the size (Fig 4.24) or proliferation (Fig 4.25) of the CMV specific $CD4^+$ T cell response.

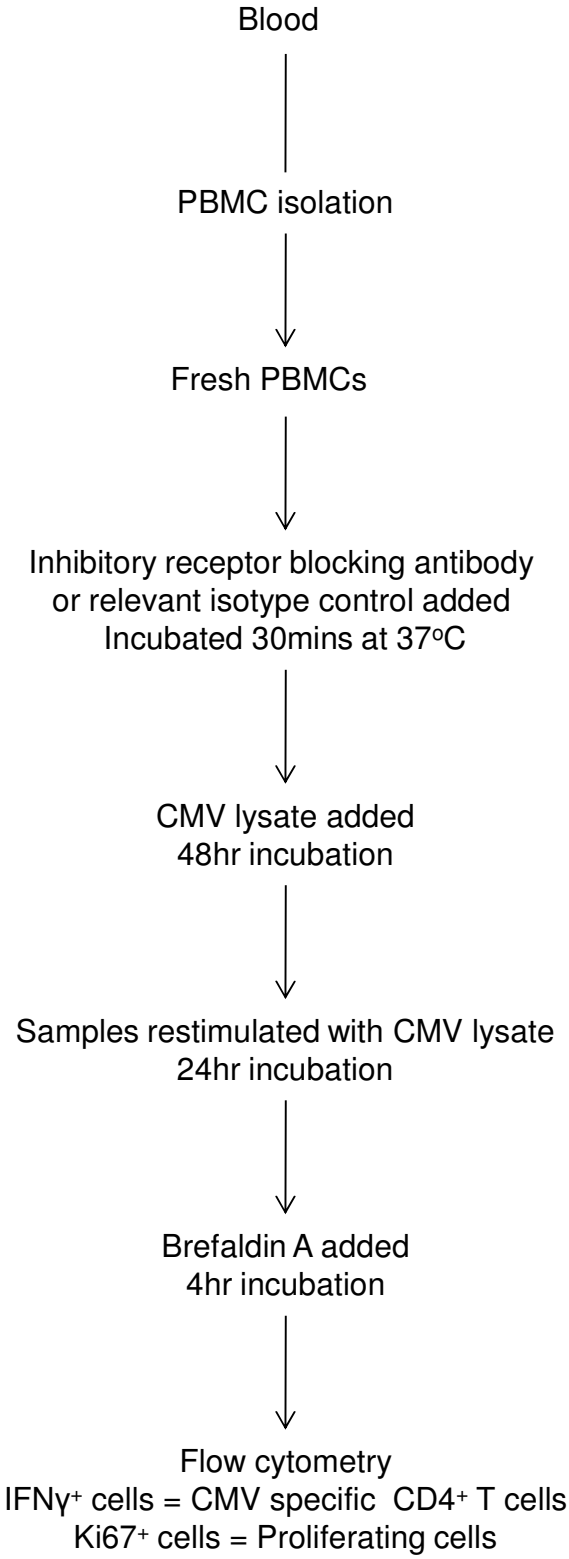


Figure 4.22 Schematic for the effects of inhibitory receptor blockade on CMV specific $CD4^+$ T cell number and proliferation experiment

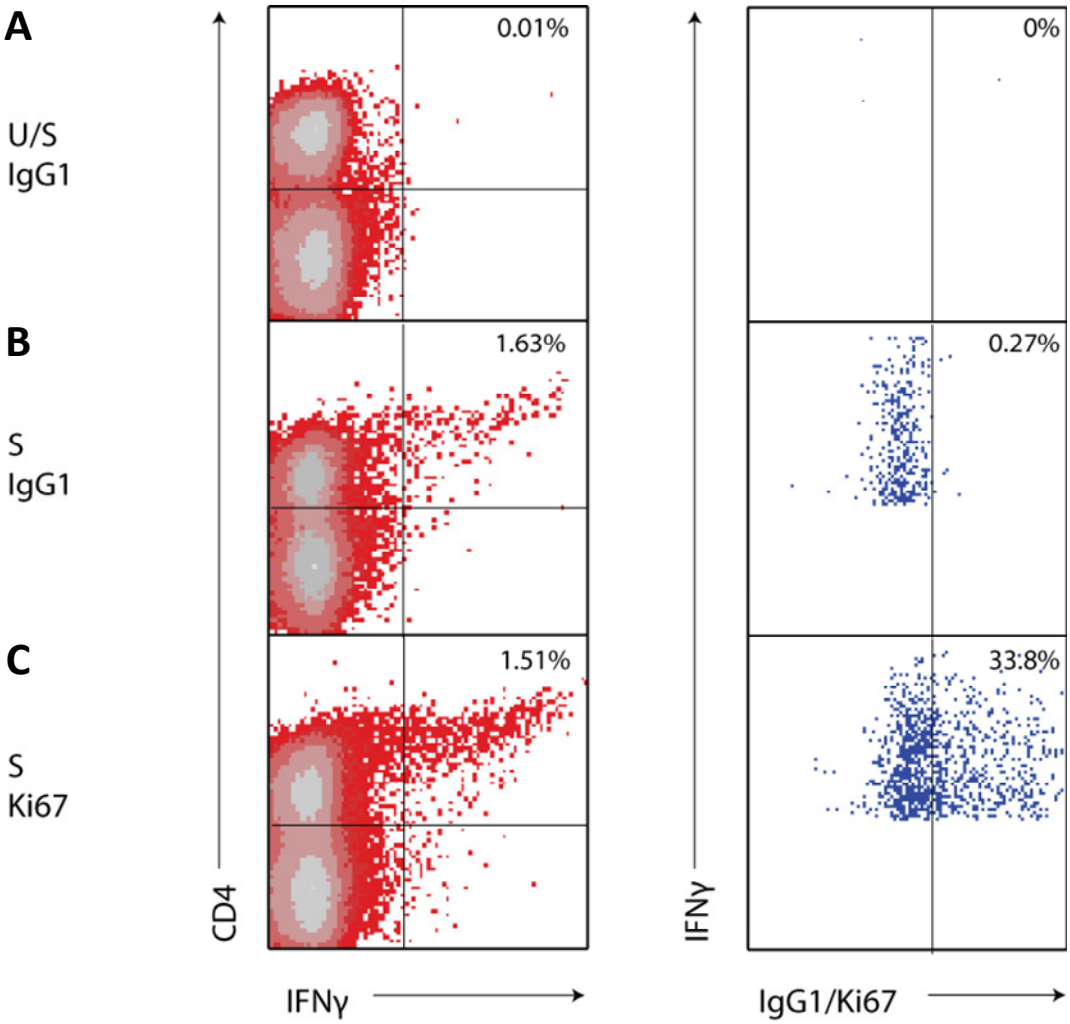


Figure 4.23. Identification of CMV specific CD4⁺ T cells and their proliferative responses.

Fresh PBMCs were incubated with CMV lysate for 3 days and re-stimulated on day 2. Cells were then stained with anti- CD4-PerCP and intranuclearly with anti- IFNγ-APC and Ki67-FITC. **(Left panels)** Representative FACS plots gated on live lymphocytes showing the proportion of CD4⁺ T cells that produce IFNγ (and were identified as CMV specific) and **(right panels)** the proportion of these IFNγ cells that express Ki67 **(A)** without and **(B)** with stimulation both following isotype staining and **(C)** following Ki67⁺ staining

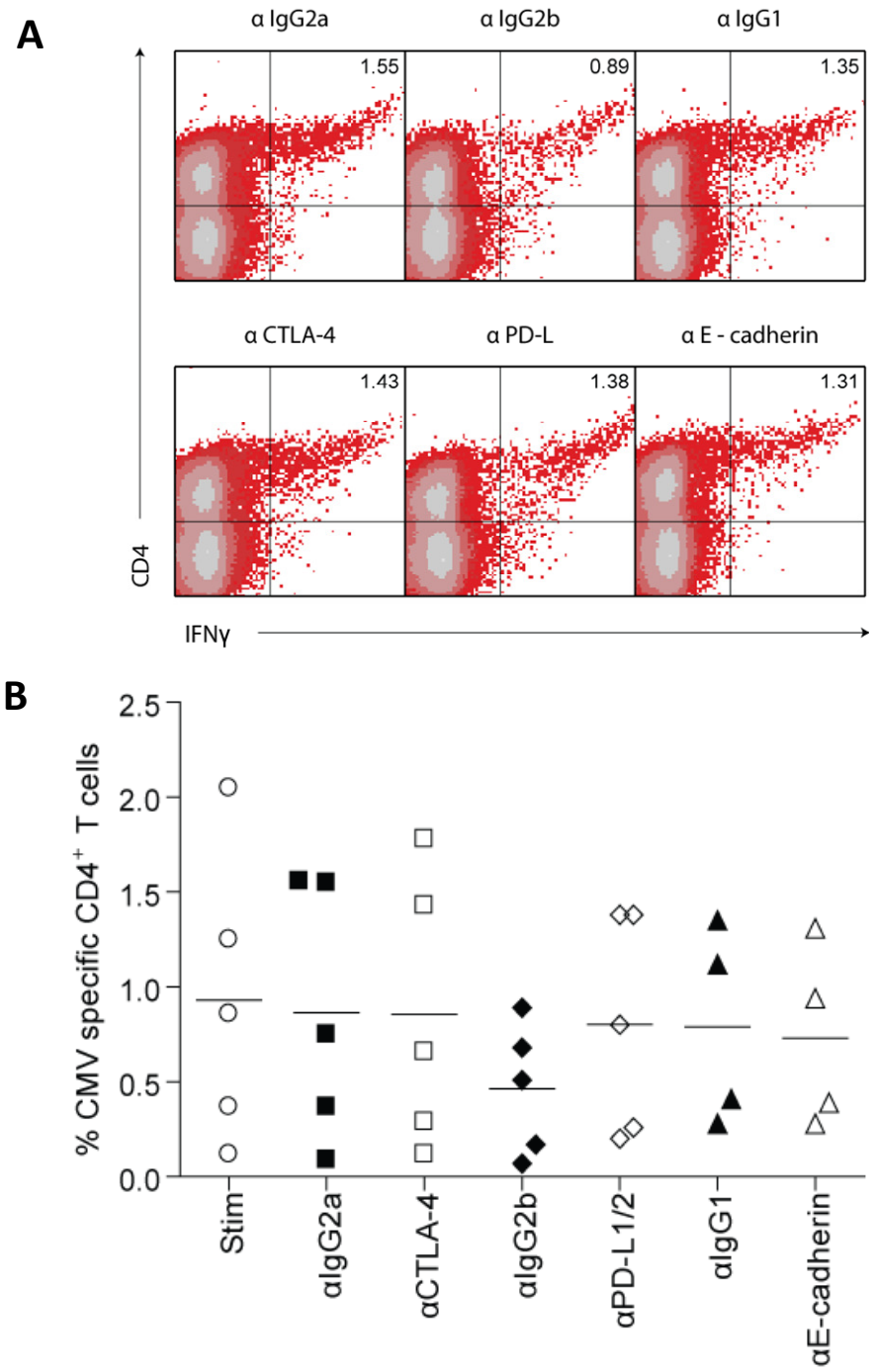


Figure 4.24. The effects of inhibitory receptor blockade on the size of CMV specific CD4⁺ T cell response.

PBMCs were stimulated with CMV lysate for 3 days and restimulated on day 2 in the presence of inhibitory receptor blockade antibody or relevant isotype control. Cells were then stained using anti- CD4-PerCP and intranuclearly with anti-IFNγ-APC and Ki67-FITC. CMV specific cells were identified as those that synthesize IFNγ. **(A)** Representative FACS plots of changes in the proportion of CD4⁺ T cells that are specific for CMV following stimulation in the presence of αCTLA-4, PD-L or E-cadherin blockade and their relevant isotype controls. **(B)** Cumulative data illustrating the effects of inhibitory receptor blockade on the proportion of CD4⁺ T cells that respond to a CMV stimulus. Horizontal lines depict mean values. The P values were assessed using a Mann-Whitney U test. Only significant differences between blocking antibody and its isotype control are shown.

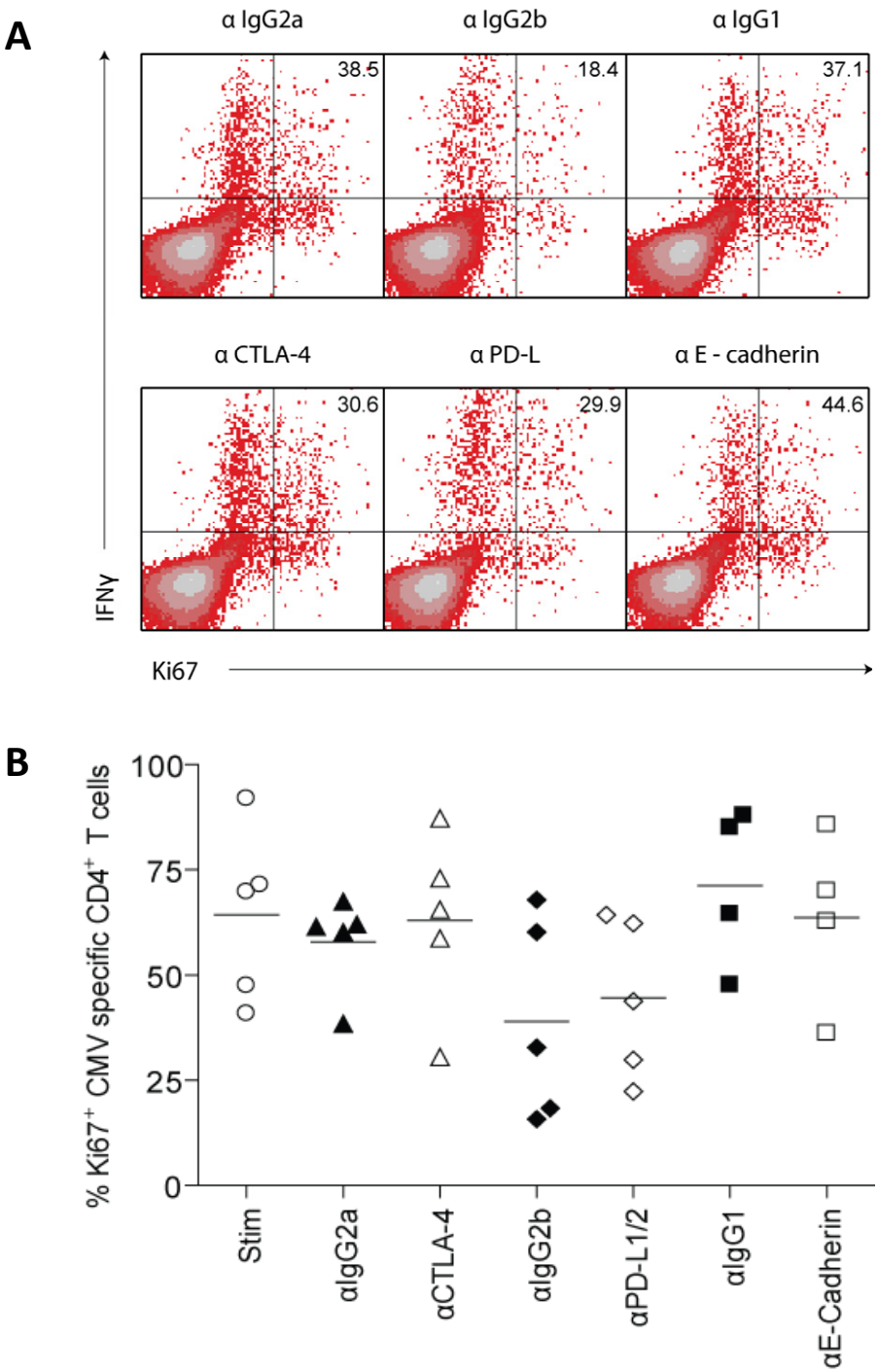


Figure 4.25. The effects of inhibitory receptor blockade on the proliferative response of CMV specific CD4⁺ T cells

PBMS were stimulated and stained as detailed in the previous figure. CMV specific CD4⁺ cellular proliferation was measured using Ki67 staining on specific cells that produce IFNγ. **(A)** Representative density dot plots, gated on CD4⁺ T cells, indicating the percentage of responding CMV specific CD4⁺ cells that express Ki67 in the presence of anti-CTLA-4, PD-L1/2 or E-cadherin blocking antibody and their relevant isotype controls. **(B)** Pooled data illustrating the effects of inhibitory receptor blockade on the proliferative response of CMV specific CD4⁺ T cells. Horizontal lines depict mean values. The P values were calculated using a Mann-Whitney U test. Only significant differences between blocking antibody and its isotype control are shown.

4.5 Discussion

Inhibitory receptor characterisation on CMV specific $CD8^+$ T cells confirmed previous reports of low CTLA-4 expression (Day et al., 2006; Kaufmann et al., 2007; Trautmann et al., 2006), mid-levels of PD-1 (Day et al., 2006; Trautmann et al., 2006; Sauce et al., 2007a; Kaufmann et al., 2007) and very high levels of KLRG1 expression on virus specific cells, which further increased with age (Ouyang et al., 2003a; Thimme et al., 2005).

Phenotypic characterisation of pp65CMV specific $CD8^+$ T cells confirmed previous findings, that expression is predominantly observed in the CD45RA-revertant memory T cell population, particularly amongst aged donors (van Leeuwen et al., 2006a). These increasingly differentiated CMV-pp65 specific $CD8^+$ T cells also increased in number with ageing, consistent with previous findings (Khan et al., 2002; Ouyang et al., 2003a; Hadrup et al., 2006). These CMV specific $CD8^+$ T cells, which we report can occupy 38% of the total $CD8^+$ T cell pool, reflect a single epitope of a single CMV protein. However, as many other CMV proteins can encode immunogenic epitopes (Sylwester et al., 2005) this figure represents an underestimation of the proportion of total $CD8^+$ T cells that are CMV specific. These large oligoclonal $CD8^+$ T cell expansions may outcompete naïve and pre-existing memory T cells for finite survival factors such as homeostatic cytokines and significantly contribute towards age onset immunodecline (Welsh and Selin, 2009).

The long standing idea, however, that the immunological space is inflexible in size (Goldrath, 2002; Freitas and Rocha, 2000) is being increasingly challenged. Recent data suggest that the T cell compartment can grow in response to antigenic challenge, such as CMV infection, and pre-existing memory cells specific for other infections are largely preserved, predicting immunity to be maintained (Vezys et al., 2009; Chidrawar et al., 2009; van Leeuwen et al., 2006b). However, a further study revealed that the functionality of this pre-existing protective immunity is nevertheless compromised (Huster et al., 2009).

Quantification of $CD4^+$ and $CD8^+$ T cell by *ex vivo* Ki67 expression also confirmed previous findings, that under steady state conditions naïve T cells turnover at a relatively poor rate. Memory T cells, on the other hand proliferate rapidly, with T_{REV}s demonstrating the lowest turnover of the memory cell subsets (Geginat et al., 2003). CMV specific $CD8^+$ T cells also exhibited low *ex vivo* proliferation that did not significantly differ from the total $CD8^+$ T cell pool, consistent with previous reports (Doisne et al., 2004; Miles et al., 2007; Champagne et al., 2001), indicating that these are resting quiescent cells.

Inhibitory receptor blocking studies revealed that PD-L blockade, but not E-cadherin or CTLA-4, enhanced the proliferative responses of CMV specific $CD8^+$ T cells. Indeed, PD-1 is also upregulated on dysfunctional HIV, HBV and HCV chronic virus specific cells and *ex vivo* PD-1/L blockade improves the functionality of these cells (Penna et al., 2007; Petrovas et al., 2006; Fisicaro et al., 2010). Thus, PD-1

upregulation may reflect a common immune evasion strategy that facilitates the establishment and maintenance of chronic infections.

Significantly, unlike $CD8^+$ T cell responses to inhibitory receptor blockade, seen in the previous chapter, the effects of PD-L blockade on CMV specific $CD8^+$ T cells were maintained amongst aged donors. However, PD-L1 has also been show to bind B7.1 at an interface that overlaps with the CD28 and CTLA-4 binding site and with an affinity higher than it binds CD28 (Butte et al., 2008). Therefore, it is possible that some functions attributed to PD-L1 blockade may reflect PD-L1 competing with CD28 for B7.1 binding. Nevertheless, the failure of anti-CTLA-4, blocking antibody, to augment CMV specific $CD8^+$ T cell responses, either individually or in combination with PD-L blockade, suggests the effects of PD-1 inhibition to be primarily mediated through interactions with PD-1.

The increased proliferative response of CMV specific $CD8^+$ T cells following PD-L blockade (IgG2b mean, 28.0%; PD-L mean, 61.8%) was not accompanied by a proportional increase in the response size (IgG2b mean, 2.75%; PD-L mean, 3.27%). This, nevertheless, concurs with PD-1 blocking studies performed in a murine LCMV model (Barber et al., 2006), suggesting that proliferation is accompanied by considerable cell death. This death may be analogous to the contraction phase of effector cell responses. Alternatively, PD-L blockade may drive the selective expansion of less exhausted cells over their more exhausted counterparts, which are driven further along the exhaustion spectrum to death (Blattman and Greenberg, 2006). Indeed, two subpopulations of virus specific $CD8^+$ T cells have been identified

in both mouse LCMV and human HCV infections: a more terminally differentiated PD-1^{hi}subset, that responds poorly to PD-1 blockade and a less exhausted more functional PD-1^{int} population that responds well to blockade (Blackburn et al., 2008; Nakamoto et al., 2008).

The inability of PD-1^{hi} virus specific $CD8^+$ T cells to respond to PD-L1 blockade may reflect the expression of additional inhibitory receptors on these highly exhausted cells (Blackburn et al., 2009). Indeed, PD-1^{hi} intrahepatic HCV specific $CD8^+$ T cells expressed CTLA-4 (unlike circulating HCV specific $CD8^+$ T cells) and responded to a combination of CTLA-4 and PD-L1 blockade, but not to the blocks individually (Nakamoto et al., 2008). Nevertheless, CTLA-4 was not expressed on LCMV or HIV specific $CD8^+$ T cells nor did CTLA-4 blockade augment their responses (Trautmann et al., 2006; Kaufmann et al., 2007; Barber et al., 2006). However, in addition to PD-1; HIV, HCV, LCMV and tumour specific $CD8^+$ T cells upregulate the inhibitory receptor: T-cell immunoglobulin domain and mucin domain 3, (Tim-3) (Jones et al., 2008; Jin et al., 2010; Fourcade et al., 2010; Sakuishi et al., 2010; Golden-Mason et al., 2009; Vali et al., 2010) and LCMV and tumour specific $CD8^+$ T cells co-express the CD4 homoloue, Lymphocyte-activation gene 3 (LAG-3) (Blackburn et al., 2009; Matsuzaki et al., 2010) and their blockade can induce functional restoration. Therefore, PD-1 may play a universal role in T cell exhaustion to persistent antigenic challenges whereas other inhibitory receptors may be may be differentially induced by different viruses, T cell subsets or anatomical locations.

The behaviour of PD-1^{inter} and PD-1^{hi} populations of CD8$^+$ T cells specific for poorly controlled persistent viral infections such as LCMV, HCV and HIV may nevertheless, not be applicable to the well controlled latent CMV infection. Indeed, an HBV study, reported that the more exhausted intrahepatic specific CD8$^+$ T cells, expressing higher PD-1 levels, responded more effectively than their less exhausted, lower PD-1 expressing, circulating counterparts (Fisicaro et al., 2010). In contrast, Nakamoto et al, (2008) documented opposing results; however in this case, the disease status of the subjects was much more advanced.

CD45RA re-expressing revertant CD8$^+$ T cells were originally named a terminally differentiated, near senescent population, as they were thought to have lost the ability to self renew and expand (Champagne et al., 2001). However, subsequently they were shown to proliferate upon challenge with specific peptide (van Leeuwen et al., 2002; Wills et al., 2002; Waller et al., 2007). HIV specific CD8$^+$ T cells from elite controllers, which are highly functional, share this typically revertant phenotype (Lichterfeld et al., 2008), in contrast to specific cells from typical HIV progressors, which are profoundly exhausted but exhibit a more intermediate differentiated phenotype (Addo et al., 2007). Nevertheless, data presented here indicate that the proliferative responses of CD45RA-revertant memory CD8$^+$ T cells are still suboptimal compared with central and effector memory populations.

Following PD-L blockade, this reduced proliferative capacity of CMV specific CD8$^+$ T_{REVS} is largely reversed. Nevertheless, we also show that PD-L blockade results in extensive phenotypic changes amongst CMV specific CD8$^+$ T cells, such that the

increased proliferative response of CD45RA-revertant memory cells may reflect differentiation changes of other subsets. Nonetheless, repeating this experiment using purified $CD8^+$ T cell subsets revealed that the revertant subset of CMV specific $CD8^+$ T cells show the strongest augmentation of proliferative responses following PD-L blockade. In contrast to results from bulk PBMCs, the proliferative response of CD45RA-revertant memory $CD8^+$ T cells, following PD-L blockade, was greater than, rather than equal to, that of their central and effector memory counterparts. This may reflect stimulated CD45RA-revertant memory $CD8^+$ T cells losing CD45RA and regaining CD45RO expression (van Leeuwen et al., 2002; Geginat et al., 2003; Wills et al., 2002).

The greatest functional effect of PD-L inhibition was found in the CD45RA-revertant memory $CD8^+$ T cell subset; this may appear at odds with expression data, showing PD-1 to be expressed at maximal levels on T_{CM} and T_{EM} populations. This dilemma may be resolved by a closer examination of the function of disrupting PD-1 signalling on different $CD8^+$ T cell populations. The blockade of PD-L on exhausted PD-1^{int} LCMV and HCV specific exhausted $CD8^+$ T cells, but not their PD-1^{hi} counterparts, restored effector functions (Nakamoto et al., 2008; Blackburn et al., 2008), while the engagement of PD-1 on resting naïve T cells, which express barely detectable levels of PD-1, effectively inhibits T cell activation (Riley, 2009). This suggests that rather than increased PD-1 expression correlating with greater signalling, differing PD-1 expression levels may engage distinct pathways (Riley, 2009). For example, PD-1 binds SHP-1 and SHP-2 in naïve T cells but in exhausted cells, the very high levels of PD-1 expression could provide more targets than SHP-1 or -2 can bind allowing the

engagement of other signalling molecules that engage distinct signalling pathways. Additionally, PD-1 may mediate distinct effects on different cell types. Indeed, modest variations in SHP-1 expression levels have been shown to switch a cell from an antigen responsive to an unresponsive state (Feinerman et al., 2008). SHP-1 levels could vary with T cell differentiation and the altered relative ratio between SHP-1 and SHP-2 may account for the distinct effects of PD-1 ligation on different T cell subsets. Furthermore, the PD-1 signalling domain contains an ITSM, which can recruit distinct signalling molecules dependent on the presence or absence of an adaptor protein (Shlapatska et al., 2001). Thus, differential expression of an as yet uncharacterised PD-1 adaptor may also play critical roles in mediating distinct effects of PD-1 signalling in different T cell subsets.

The question remains that if PD-L blockade has the greatest effects on revertant $CD27^{-}CD45RA^{+}$ $CD8^{+}$ T cells, why did it not significantly augment the response of highly differentiated $CD27^{-}CD28^{-}$ $CD8^{+}$ T cells to an anti-CD3 stimulus? Conversely, E-cadherin blockade was shown to augment the proliferative responses of highly differentiated $CD8^{+}$ T cells but, contrary to what might have been expected, did not have a similar effect on CMV specific $CD8^{+}$ T cells. This may be a consequence of the different stimuli being used with the anti-CD3 stimuli reflecting an acute model of activation and, indeed, PD-$L1^{-/-}$ mice make responses analogous to WT towards an acute LCMV strain but succumb to a chronic strain (Barber et al., 2006) and also KLRG1 is upregulated during acute but not chronic LCMV infections (Wherry et al., 2007). Alternatively, the revertant $CD27^{-}CD45RA^{+}$ CMV specific $CD8^{+}$ T cell population may not be analogous to the highly differentiated $CD27^{-}CD28^{-}$ $CD8^{+}$ T

cell subset. Nevertheless, in both these models, the simultaneous blockade of PD-1, CTLA-4 and KLRG1 did not result in any additional or synergistic effects, reflecting that these inhibitory receptors feed into a common PI3K/Akt signalling pathway (Parry et al., 2005; Tessmer et al., 2007; Henson et al., 2009).

Unlike $CD8^+$ T cells, PD-1/L blockade does not augment CMV specific $CD4^+$ T cell proliferative responses, which is consistent with previous findings (Kaufmann et al., 2007; Sester et al., 2008; Kassu et al., 2010) and may reflect CMV specific $CD4^+$ T cells being exhausted to a much more modest extent than $CD8^+$ T cells or, alternatively, CMV specific $CD4^+$ and $CD8^+$ T cells may be subject to distinct mechanisms of immune regulation, as observed in HIV infection (Kaufmann et al., 2007).

In summary, PD-1/L blockade can augment the proliferative response of CMV specific $CD8^+$ T cells, however, it is unclear as to whether this reflects true reversal of exhaustion and cellular ageing processes or whether it simply increases turnover that may further exhaust cells already close to replicative senescence. This idea is investigated in chapter 5 by examining whether PD-1/L blockade upregulates telomerase expression in CMV specific $CD8^+$ T cells.

5 Determination of the Role of CMV Infection and Immune Inhibitory Receptors in Age-associated Telomere Attrition

5.1 Introduction

The impairment of telomerase upregulation and resultant lymphocyte telomere attrition are critically implicated in age onset immune decline (see chapter 1.2.3.2.3). However, the effects of CMV infection on T cell telomere length have yet to be fully explored and will be investigated in this chapter. Moreover, CD28 is required for the induction of telomerase activity through Akt, which phosphorylates and activates the catalytic component of telomerase, hTERT (Akbar and Vukmanovic-Stejic, 2007) and CTLA-4, PD-1 and KLRG1 all inhibit CD28 signalling (see section 1.5.4.3). Therefore, their increased expression on T cells with age and/or CMV status may be responsible for driving age-associated telomere attrition and associated immunosenescence and this hypothesis is explored in the current chapter.

5.2 Measuring telomere lengths using the Flow-FISH assay

Lymphocyte telomere lengths were determined using a 3 colour flow-FISH assay. A representative dot plot depicting a FSC/SSC profile following flow-FISH, with a live lymphocyte gate depicted (Fig 5.01A). A representative CD4 and telomere probe stain, gated on live lymphocytes (Fig 5.01B), with the small proportion of cells not uptaking the probe indicated. To counteract the effect of these outliers, the median value of the telomere probe intensity was recorded on the relevant population (representative

example gated on $CD4^+$ T cells Fig 5.01C). This telomere median fluorescence intensity was converted into telomere length using a standard curve generated in the laboratory (Fig 5.01D). Two cryopreserved PBMC samples of known telomere length and fluorescence were also used as instrument controls to ensure consistency of results.

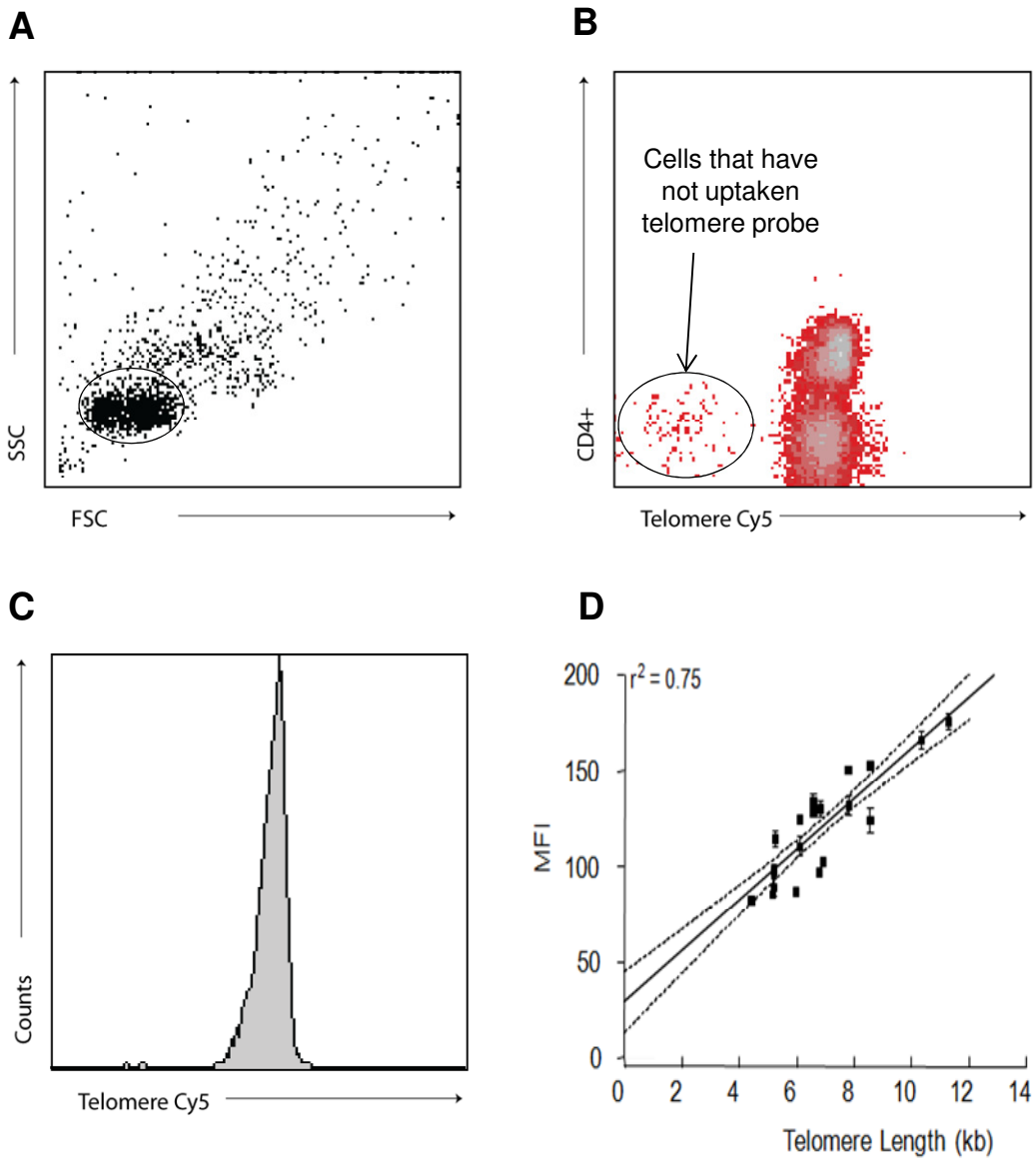


Figure 5.01. Measurement of telomere lengths by flow-FISH

The telomere length of total lymphocytes, as well as $CD8^+$, $CD4^+$ and $CD4^-CD8^-$ lymphocyte subpopulations was measured using a 3 colour flow-FISH (flow cytometry in combination with fluorescence in situ hybridization) assay with anti-CD8-FITC, CD4-biotin and a telomere Cy5 probe which was then subjected to flow cytometric analysis. **(A)** Representative dot plot of FSC/SSC profile with gate for live lymphocytes displayed. **(B)** FACS plot gated on live lymphocytes demonstrating telomere probe staining against CD4 expression. Indicated are a minority of cells hat have not uptaken the telomere probe. **(C)** Histogram of telomere probe staining on $CD4^+$ T cells and the median value is measured rather than the mean to prevent these outliers skewing measurement of the distribution. **(D)** Telomere MFI could be converted into telomere length in kilobases using a standard curve. Two cryopreserved PBMC samples of known telomere fluorescence used as standards to ensure consistency of results

5.3 CMV accelerates age-associated telomere attrition in T cell populations

5.3.1 Live lymphocytes

The telomere lengths of total live lymphocyte populations strongly negatively correlated with age, both in CMV^- and CMV^+ cohorts (Fig 5.02A). However, this diminution was exacerbated amongst CMV^+ donors, losing an average of 61 base pairs per year (bpy) compared to 45 bpy amongst CMV^-s, such that CMV^+ old individuals have significantly shorter telomeres than aged CMV^- donors, whereas no such difference is observed in the young (Fig 5.02B).

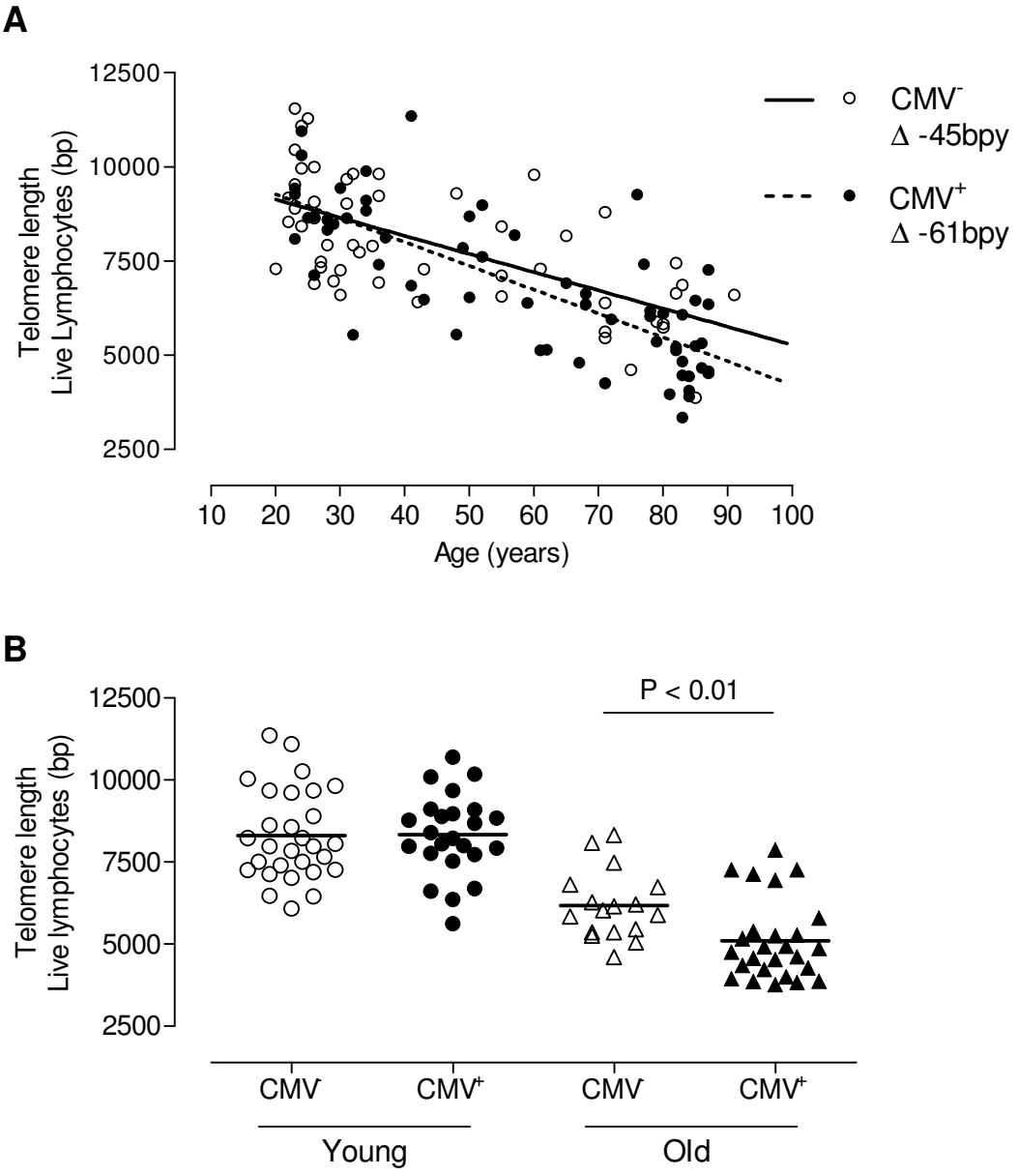


Figure 5.02 CMV accelerates telomere attrition in total lymphocytes from healthy individuals.

The telomere length was measured as described in the previous figure. **(A)** Correlation between telomere length and age in total lymphocytes (CMV negative: n=56, r^2=0.41, P<0.0001; CMV positive: n=58, r^2=0.58, P<0.0001), from CMV positive and negative healthy individuals. The rate of telomere attrition, represented as loss of base pairs per year (bpy) was calculated from the gradient is indicated. Open circles and an unbroken line represent CMV negative individuals, filled circles and a dashed line represent CMV positive individuals. Line of best fit generated using linear regression. r^2 and P values generated using Pearson's correlation. **(B)** Telomere lengths of these individuals is represented by grouping via age (young < 40 years, old > 70) and CMV status. Horizontal bars depict mean values and Students t test was used to calculate P values, only significant differences between age matched CMV^- and CMV^+ individuals are shown.

5.3.2 CD8$^+$ T cells

The rate of age-associated telomere attrition was greatest amongst CD8$^+$ T cells and was further accelerated by CMV infection (Fig 5.03A, CMV$^+$: -94bpy, CMV$^-$: -72bpy). Old CMV$^+$ donors exhibited significantly shorter telomeres than their CMV$^-$ counterparts (Fig 5.03B).

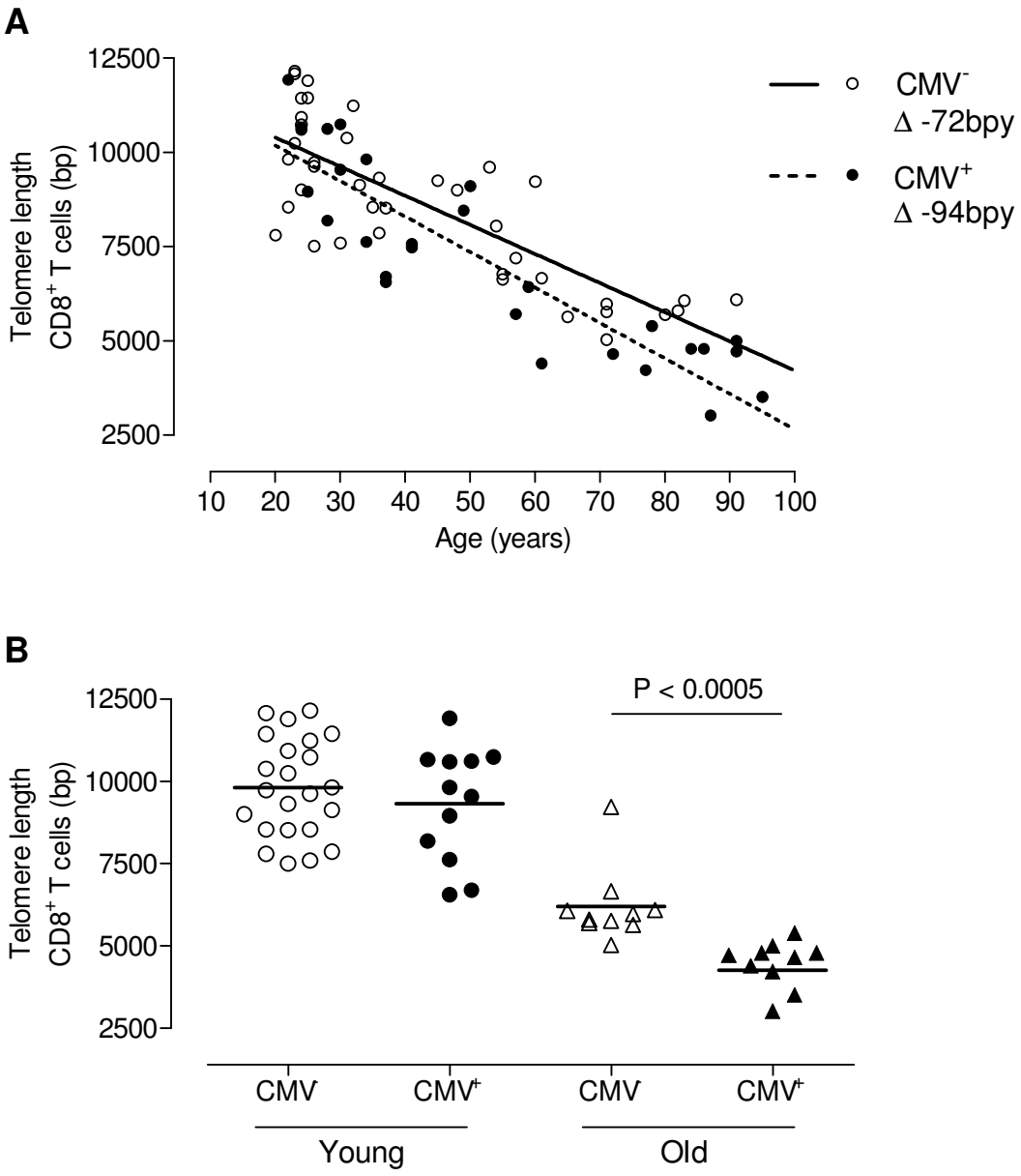


Figure 5.03. CD8+ T cell telomere loss is accelerated in CMV infected as compared to CMV free donors

Telomere lengths of CD8+ T cells was determined as described in Fig 5.01. **(A)** CD8+ telomere lengths of healthy donors correlated by age and CMV status (CMV negative: n=40, r^2=0.63, P<0.0001; CMV positive: n=29, r^2=0.79, P<0.0001). The gradient was used to calculate the rate of telomere attrition as expressed in base pairs per year. Open symbols represent CMV- donors and filled symbols depict CMV+ subjects. Line of best fit generated using linear regression. r^2 and P values generated using Pearson's correlation. **(B)** CD8+ T cell telomere lengths of CMV- and CMV+ individuals compared by grouping them into either young (<40) or old (>60 years) and performing Student's test with only significant differences between CMV+ and CMV- age matched donors indicated.

5.3.3 CD4$^+$ T cells

Similarly, CMV infection accelerated donor telomere loss in CD4$^+$ T cells (Fig 5.04, CMV$^+$: -65bpy; CMV$^-$: -47bpy) with CMV$^+$ old possessing significantly reduced telomeres than observed for CMV$^-$ old donors (Fig 5.04B).

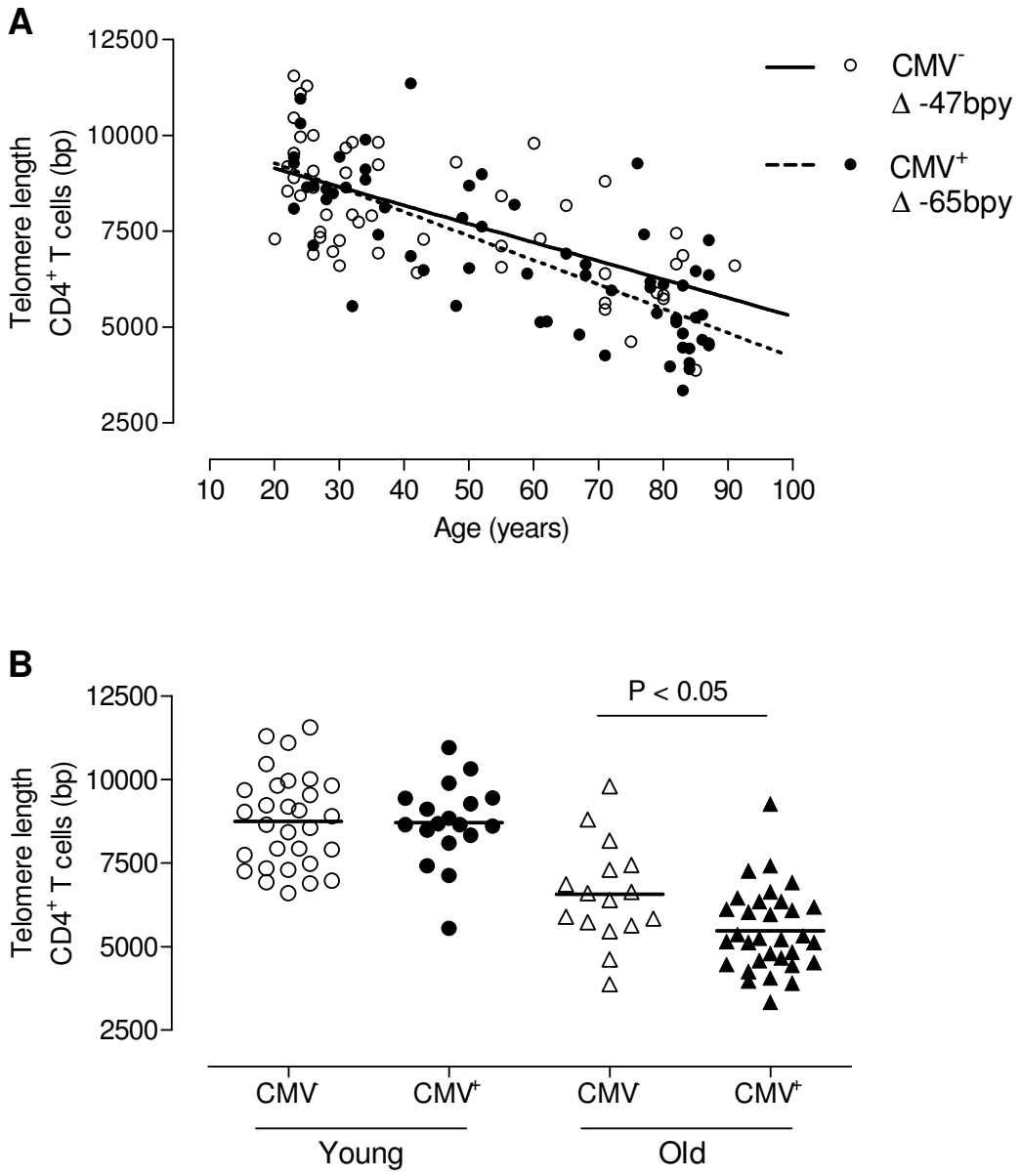


Figure 5.04. Age associated telomere shortening of CD4+ T cells is accelerated in CMV+ donors

$CD4^+$ T cell telomere lengths were determined in accordance with the protocol described in Fig 5.01. **(A)** Scatter plots illustrating telomere lengths of $CD4^+$ T cells from donors, stratified by age and CMV status (CMV negative: n=52, r^2=0.41, P<0.0001; CMV positive: n=64, r^2=0.58, P<0.0001) with the average rate of telomere attrition in both CMV^- and CMV^+ cohorts indicated. Linear regression was used to generate line of best fit and Pearson's correlation generated r^2 and P values. **(B)** Column graphs depicting the $CD4^+$ T cell telomere lengths amongst CMV^- and CMV^+ individuals within young and old cohorts. Open symbols represent CMV^- and closed symbols depict CMV^+ donors. Mean values are depicted by horizontal bars and P values calculated using Student's t test. Only significant differences between CMV infected and CMV free donors of the same age cohort are depicted.

5.3.4 $CD4^-CD8^-$ Lymphocytes

CMV did not exhibit any acceleration in telomere attrition in $CD4^-CD8^-$ lymphocytes (Fig 5.05A, CMV^-: -43bpy, CMV^+: -46bpy) and CMV^+ and CMV^- donors do not significantly differ in their telomere lengths amongst young and old donors (Fig 5.05B)

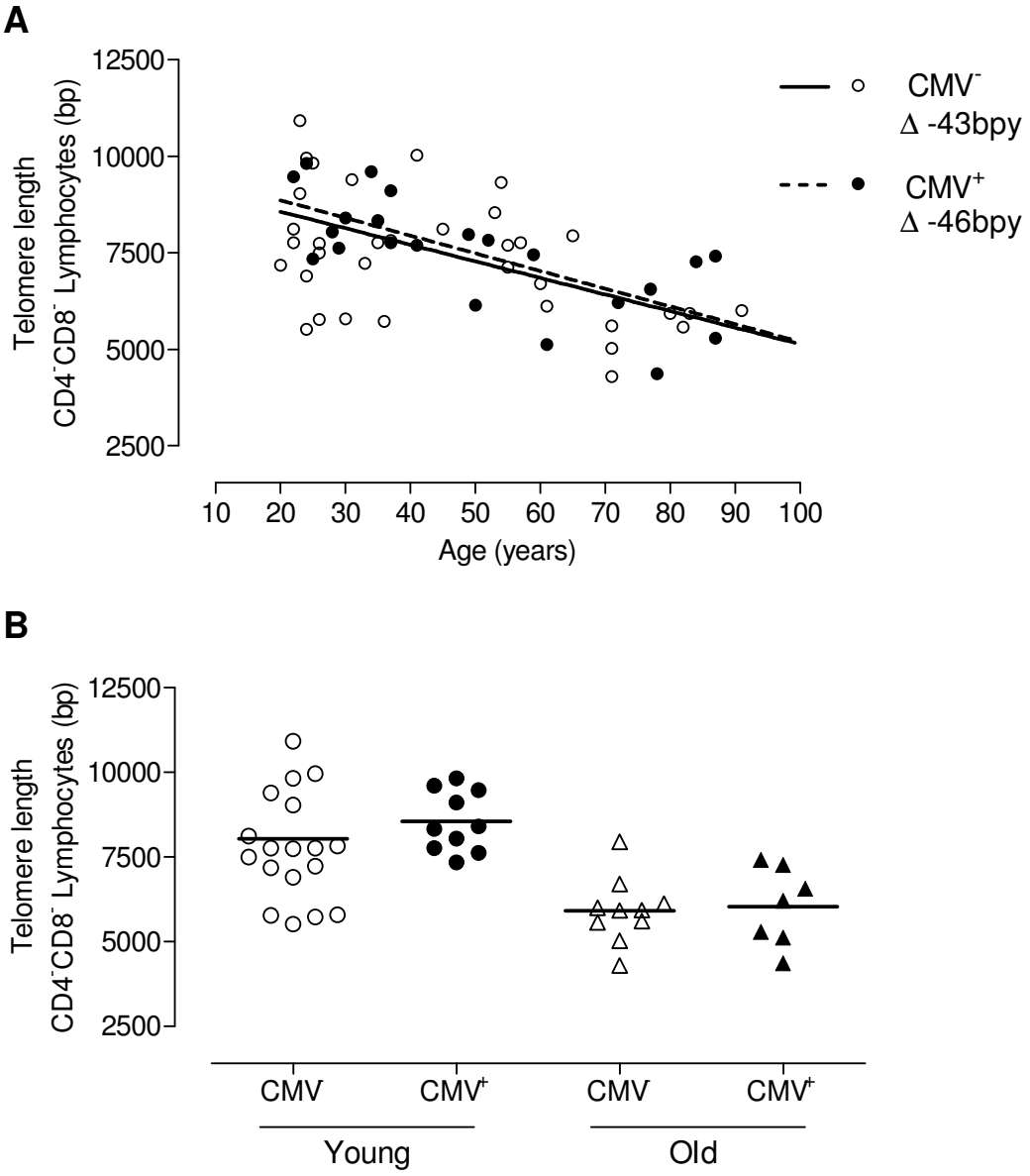


Figure 5.05. CMV does not accelerate telomere attrition in $CD4^-CD8^-$ T cells populations from healthy individuals.

Flow-FISH was performed in accordance with Fig 5.01 to determine telomere lengths of $CD4^-CD8^-$ lymphocytes. **(A)** Scatter plots illustrating how the telomere lengths of $CD4^-CD8^-$ lymphocytes change with age and CMV status of donors. (CMV negative: n=36, r^2=0.26, P<0.005; CMV positive: n=22, r^2=0.50, P<0.0005). Linear regression was used to produce lines of best fit, from which the rates of telomere loss in CMV^- and CMV^+ donors, expressed as base pairs per year (bpy), was calculated. **(B)** Telomere lengths of $CD4^-CD8^-$ lymphocytes comparing in CMV^- compared with CMV^+ subjects in both young and old donors are displayed in a column graph with student's t test used to calculate P values. Only significant differences are displayed. Filled symbols represent CMV^+ and open symbols depict CMV^- donors.

5.4 Correlating telomere length with inhibitory receptor expression

5.4.1 CTLA-4

CTLA-4 expression on $CD8^+$ T cell exhibited a strong highly significant negative correlation with their telomere lengths (Fig 5.06A) both amongst CMV^- and CMV^+ donors (Fig 5.06B). However, amongst $CD4^+$ T cells, CTLA-4 expression exhibits a weak, negative correlation with their telomere lengths that does not reach significance irrespective of CMV status (Fig 5.07).

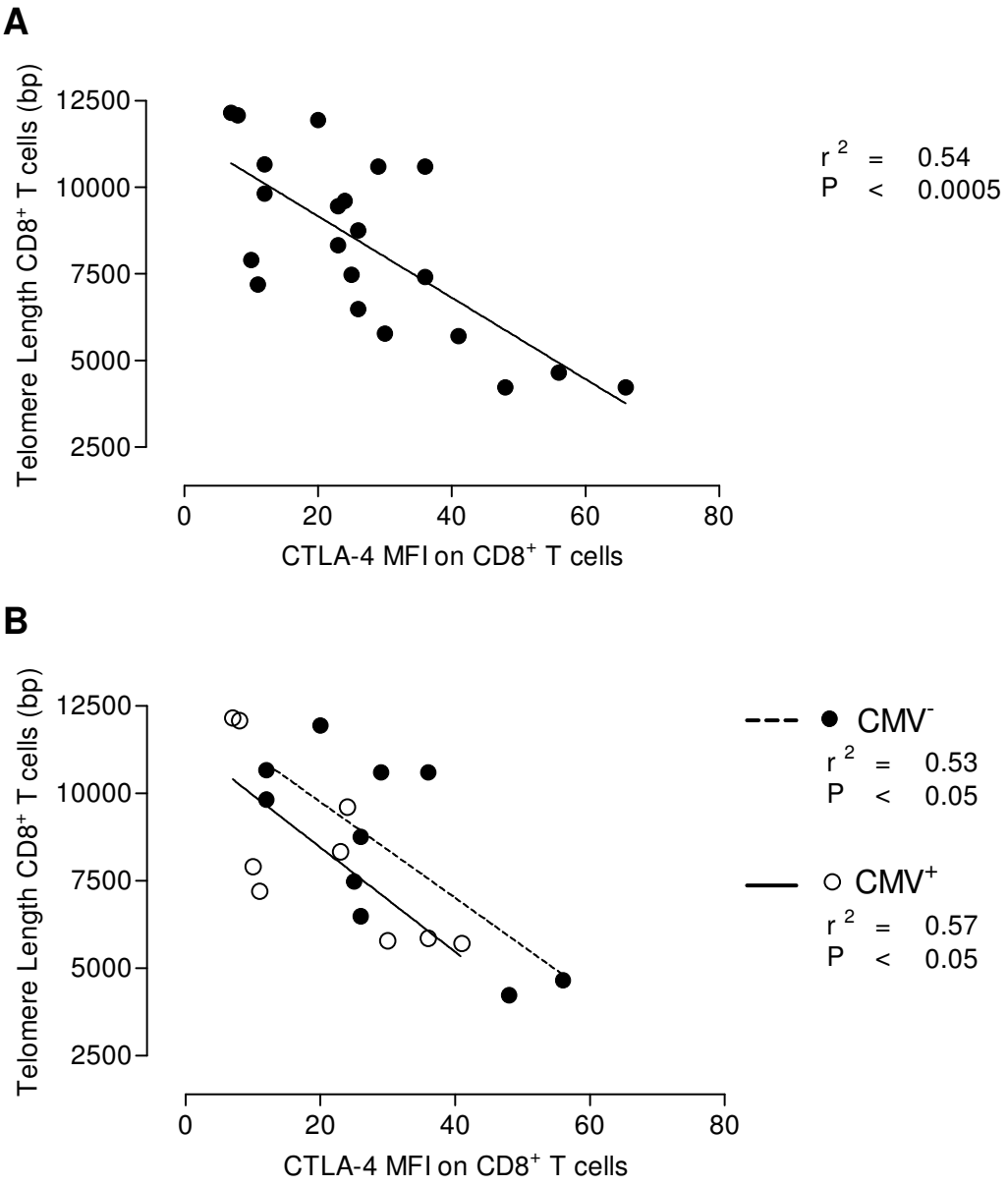


Figure 5.06. Telomere lengths of CD8+ T cells varies with their CTLA-4 expression

PBMCs were stimulated for 24hrs with anti-CD3 before staining with anti- CD8-FITC, CD4-PerCP and intracellularly with CTLA-4-PE to determine CTLA-4 expression on CD8+ T cells. The telomere length of these donors was assessed using Flow-FISH as described earlier. **(A)** Correlation between CTLA-4 expression and length of telomeres on the CD8+ T cell pool of different individuals. **(B)** Relationship between CD8+ T cell telomere lengths and their CTLA-4 expression of CMV negative (unfilled circles) and CMV positive (filled circles) donors. Line of best fit generated using linear regression. P and r^2 values generated using Pearson's correlation.

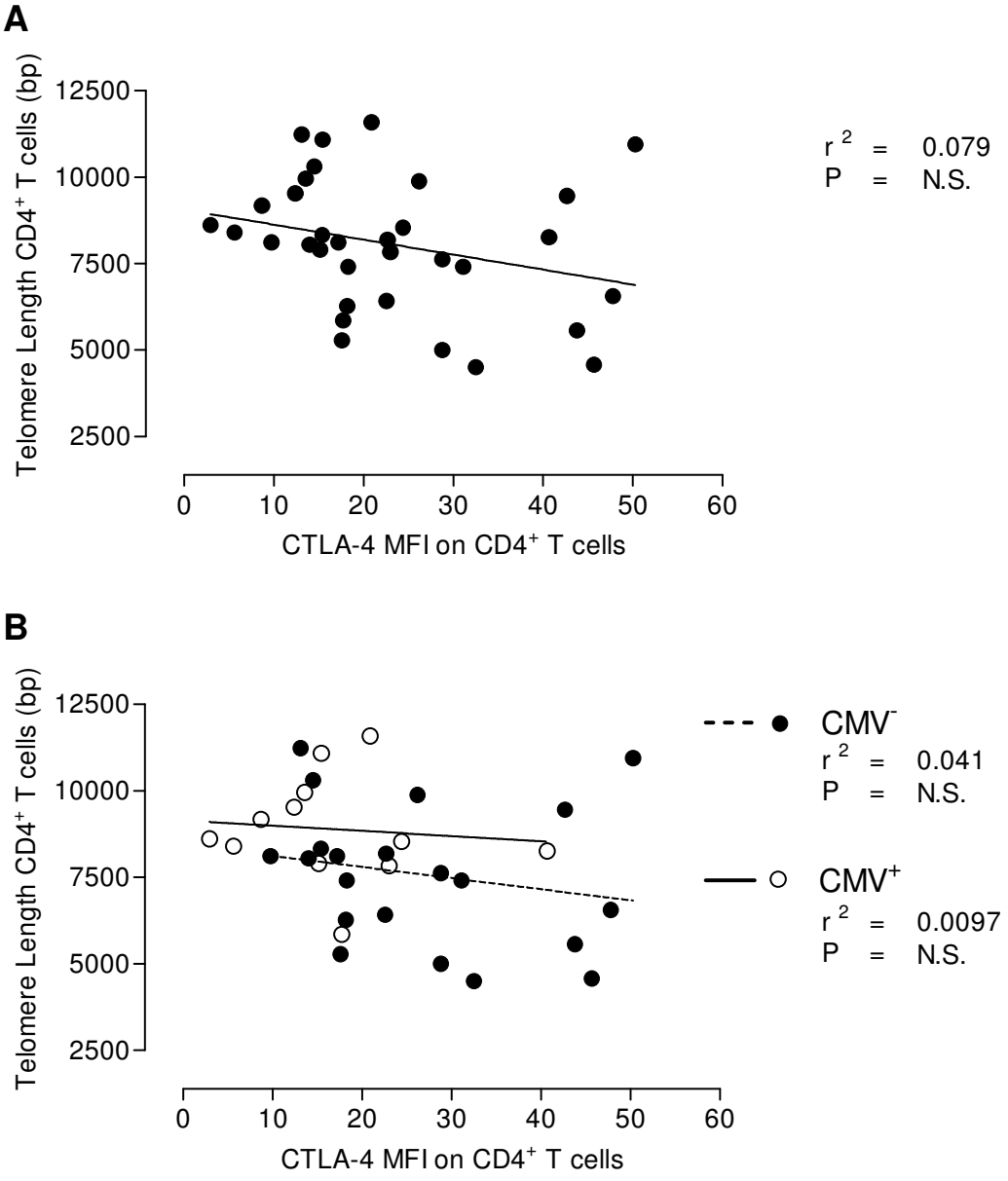


Figure 5.07 The relationship between CTLA-4 expression of CD4+ T cells and their telomere lengths

PBMCs were stained and analysed as described in the previous figure**. (A)** Scatter plot depicting variation of telomere lengths of CD4+ T cells with their CTLA-4 expression **(B)** CD4+ T cell telomere lengths as stratified by their CTLA-4 expression and donor CMV status. Line of best fit generated using linear regression. Open circles indicate CMV- donors and filled symbols depict CMV+. P and r^2 values generated using Pearson's correlation.

5.4.2 PD-1

Telomere lengths of $CD8^+$ T cells did not exhibit a significant correlation with their PD-1 expression on total, CMV^- or CMV^+ donors (Fig 5.08). The same result was observed for $CD4^+$ T cells (Fig 5.09).

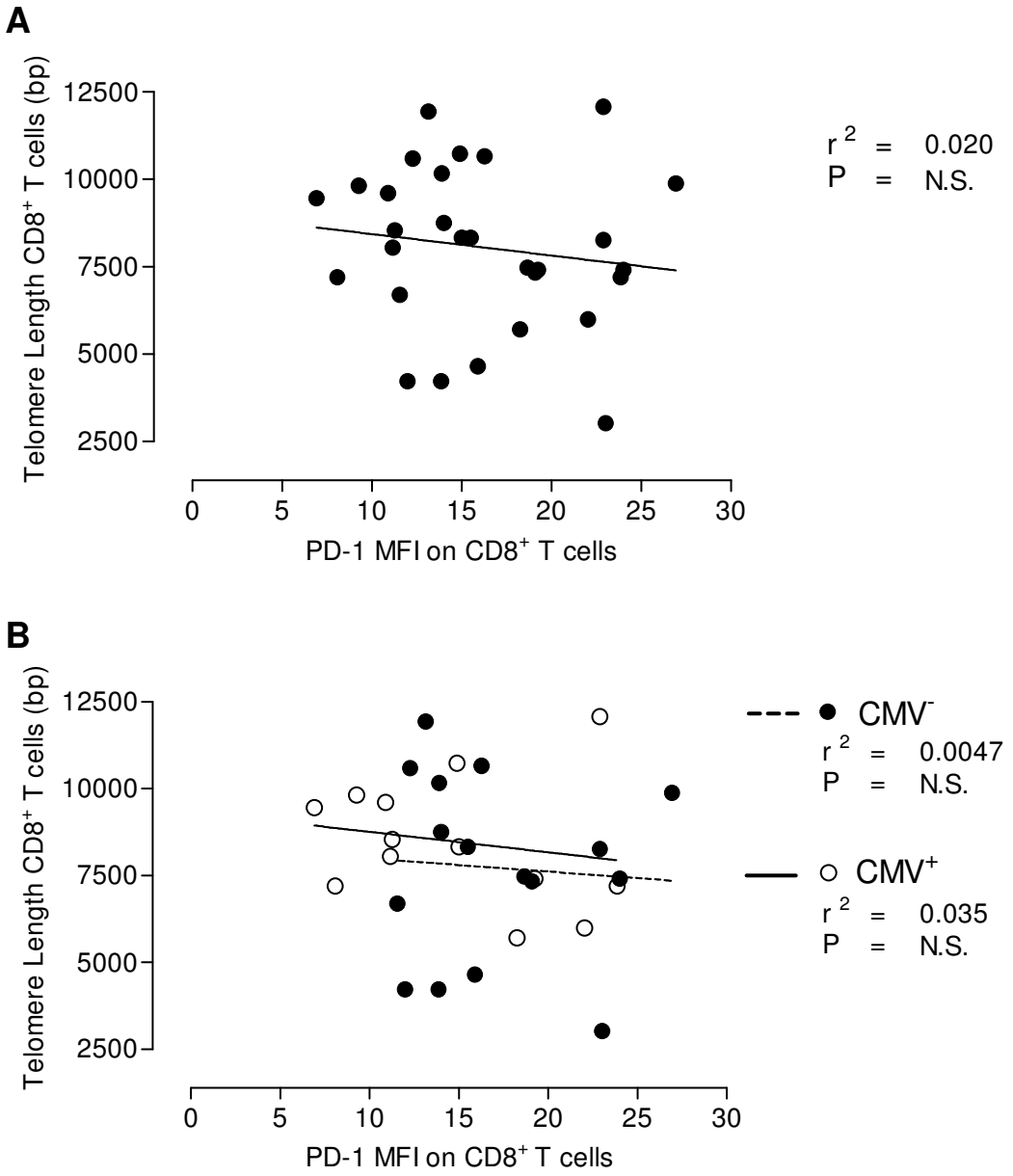


Figure 5.08 Correlation between PD-1 expression and telomere lengths of CD8⁺ T cells.

PBMCs stimulated for 48hr and then stained with anti- CD8-FITC, CD4-PerCP and PD-1-PE and analysed by flow cytometry. Telomere lengths using PBMCs from the same donor was determined, as stated in Fig 5.01. **(A)** Correlation between PD-1 expression and length of telomeres in their CD8⁺ T cell pool. **(B)** Telomere lengths of CD8⁺ T cells was stratified by their PD-1 expression on CMV negative (unfilled circles, dashed line) and positive (filled circles, solid line) donors. Line of best fit generated using linear regression. Pearson's correlation was used to generate r^2 and P values

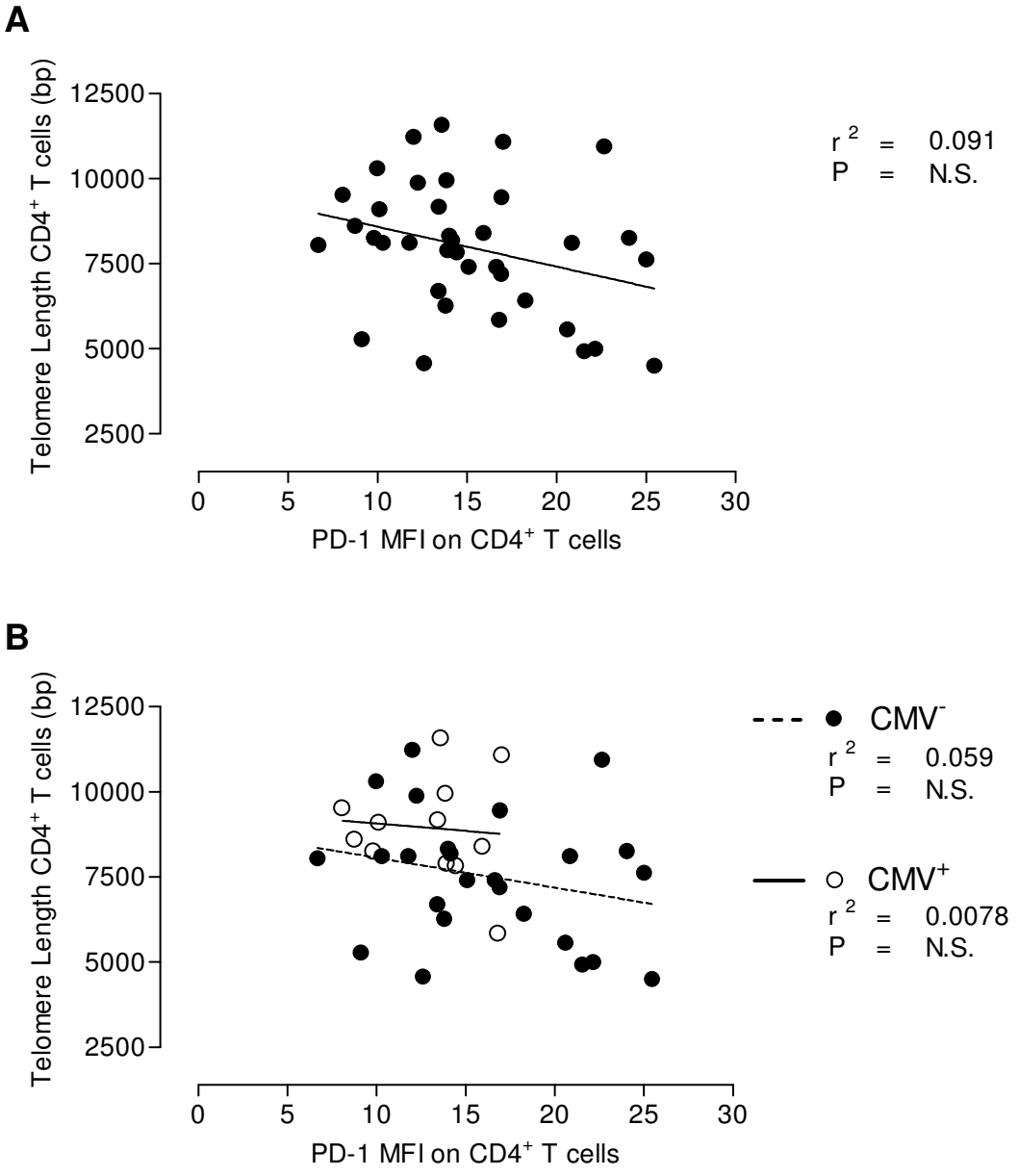


Figure 5.09 Differences in telomere lengths of CD4+ T cells as stratified by PD-1 expression in CMV- and CMV+ donors.

PBMCs stimulated and stained as described in the previous figure. **(A)** CD4+ T cell telomere lengths, as correlated by PD-1 expression and **(B)** further stratified based on donor CMV status. Line of best fit generated using linear regression. CMV+ donors depicted by filled circles and broken line whereas the solid line and open symbols depict CMV- donors. R^2 and P values generated using Pearson's correlation.

5.4.3 KLRG1

The KLRG1 expression of $CD8^+$ T cells could be strongly and negatively correlated with their telomere lengths on all donors (Fig 5.10A) and also after stratification for CMV status (Fig 5.10B). However, the telomere lengths of $CD4^+$ T cells could only be weakly correlated with their KLRG1 expression in a manner that does not reach significance (Fig 5.11A). However, whereas CMV^+ individuals display a non-significant weak negative correlation, the $CD4^+$ T cell telomere lengths of CMV^- donors appears to be independent of their KLRG1 expression (Fig 5.11B).

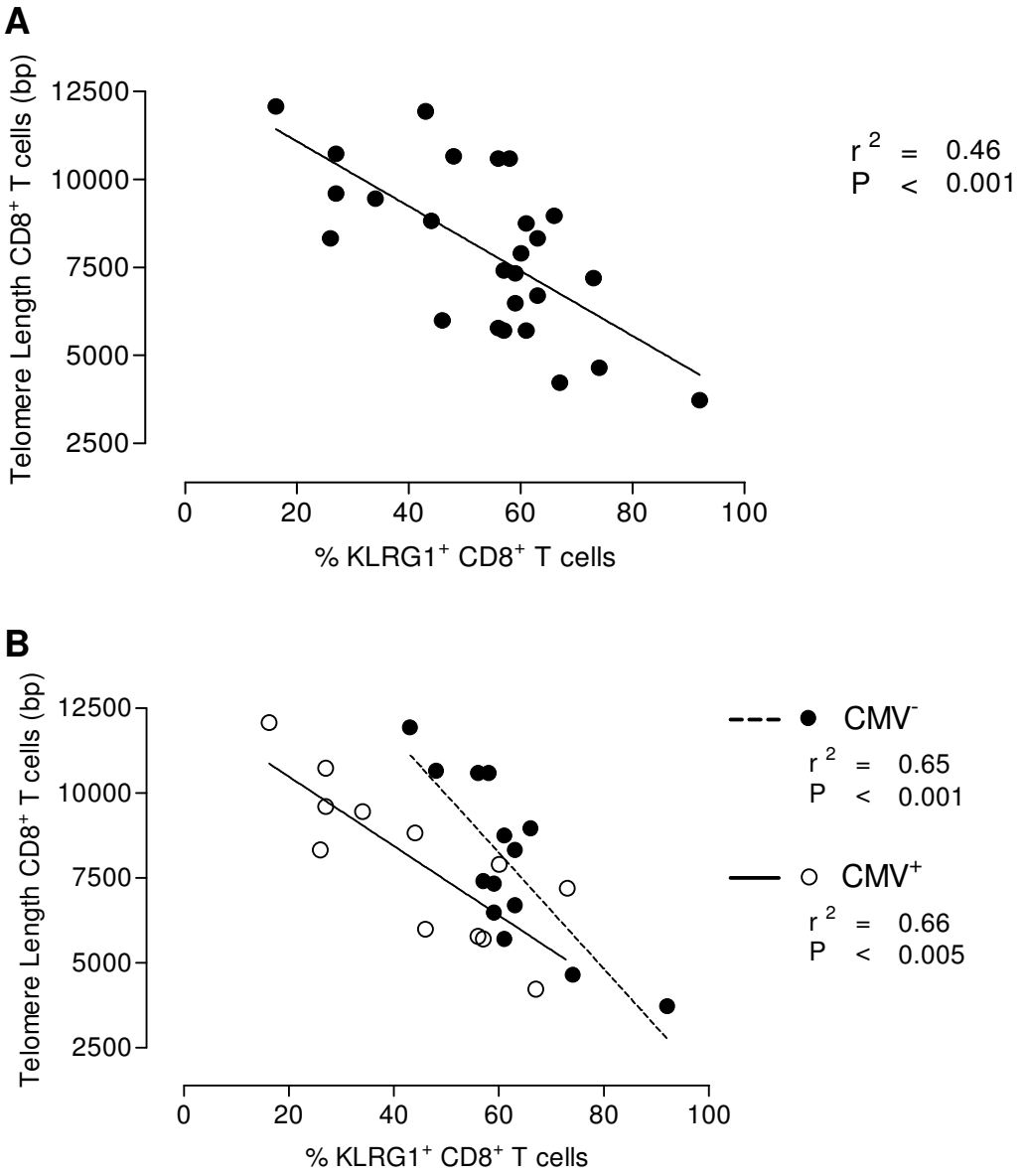


Figure 5.10 KLRG1 expression negatively correlates with telomere lengths in CD8+ T cells

PBMCs stained with anti- KLRG1-PE and CD8-FITC and their telomere lengths determined as described in Fig 5.01. **(A)** Telomere lengths of $CD8^+$ T cells (y-axis) is plotted against their KLRG1 expression (x axis) and **(B)** further stratified on the basis of donor CMV status. Open circles represent CMV^- and filled circles CMV^+ donors. R^2 and P values generated using Pearson's correlation and the line of best fit was generated using linear regression.

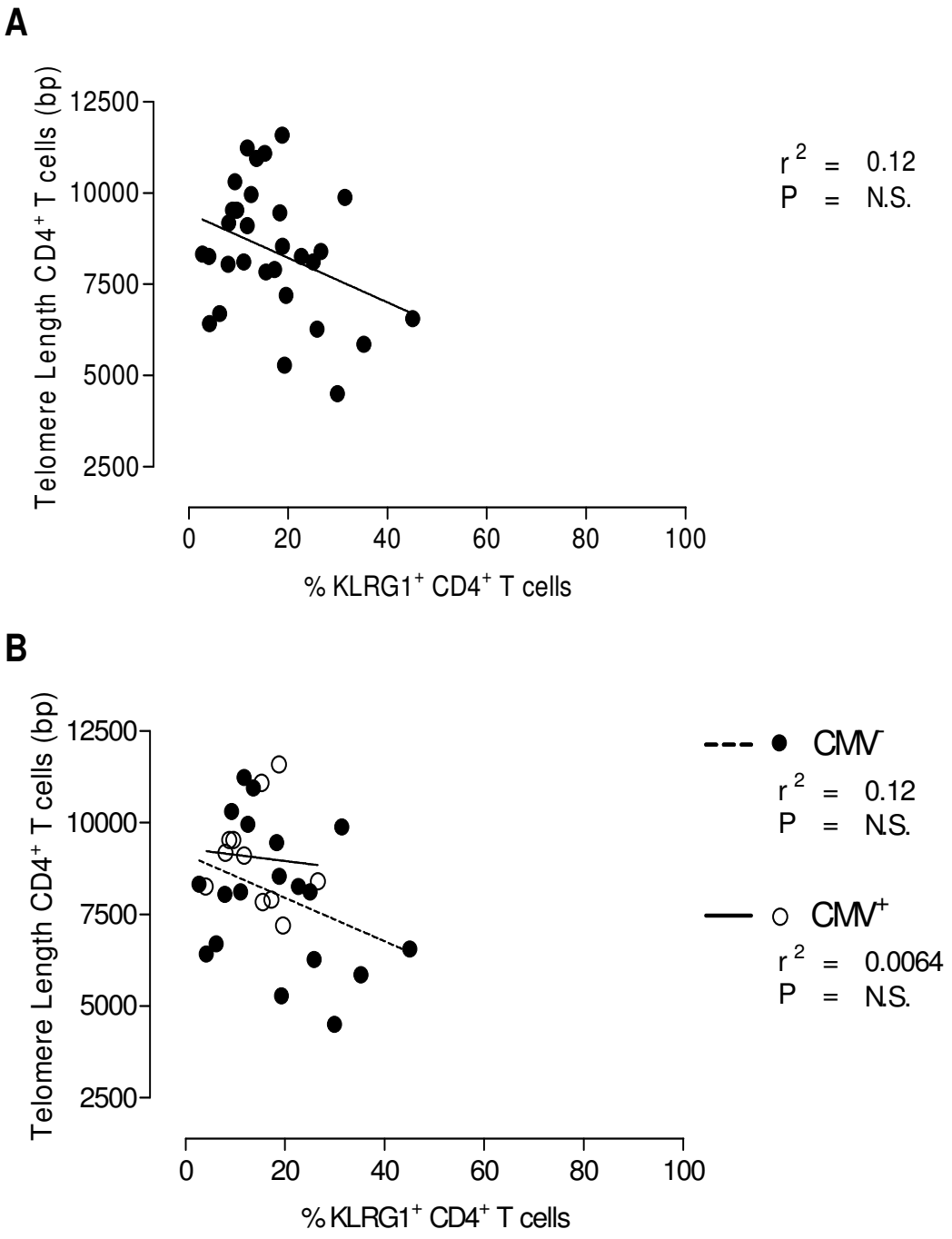


Figure 5.11 Telomere lengths of CD4+ T cells correlated by their KLRG1 expression

PBMCs stained and analysed as described in the previous figure. **(A)** Correlation between KLRG1 expression and length of telomeres on the CD4+ T cell pool of different individuals. **(B)** CD4+ T cell telomere lengths stratified by their KLRG1 expression on CMV negative (unfilled circles, filled line) and CMV positive (filled circles, dashed line) donors. Line of best fit generated using linear regression. R^2 and P values generated using Pearson's correlation

5.5 Functionally defining inhibitory receptor expression in mediating telomerase defects of highly differentiated bulk and CMV specific $CD8^+$ T cells

5.5.1 Blocking inhibitory receptor interactions with their ligands does not augment telomerase expression of $CD8^+$ T cells at any stage of their differentiation

Ex vivo $CD8^+$ T cells at different stages of differentiation, from 3 different donors, were purified and stimulated with anti-CD3 and autologous irradiated APCs in the presence of inhibitory receptor blockade or their relevant isotype control. Following a 3 day stimulation the cells were stained with Ki67 and a telomerase ELISA was performed using samples adjusted to 500 $Ki67^+$ cells per reaction. However, blocking CTLA-4, PD-L or E-cadherin did not increase the $CD8^+$ T cell telomerase activity at any differentiation state (data not shown).

5.5.2 CMV specific $CD8^+$ T cell telomerase activity was not upregulated following PD-L blockade

Whether the increased proliferative capacity seen in CMV specific $CD8^+$ T cell following PD-L blockade represents true reversal of exhaustion (Chapter 4) was determined by investigating if this proliferative increase was accompanied by telomerase upregulation. This was accomplished by repeating the above experiment but using a pp65 CMV peptide pool instead of an anti-CD3 stimulus. A pp65CMV peptide pool was used as NLV or TPR peptides did not induce a sufficiently large Ki67 expression to perform the telomerase assay. Blocking PD-1/L interactions

significantly increased the proliferative responses of $CD8^+$ T cells stimulated with pp65, in a manner analogous to an NLV/TPR peptide stimulus, (data not shown, $P < 0.005$). However, despite this increase in proliferation, blocking PD-1 interactions did not upregulate the telomerase activity of $CD8^+$ T cells at any differentiation state (data not shown).

5.6 Discussion

Data presented here confirm previous reports of age-associated telomere attrition among live lymphocytes (Slagboom et al., 1994), $CD8^+$ and $CD4^+$ T cells (Weng et al., 1995; Rufer et al., 1999) of healthy donors. The $CD4^-CD8^-$ T cell population also displayed age related telomere diminution and likely comprises a mixture predominantly of NK and B cells, which have also been shown to shorten their telomeres with increasing age (Mariani et al., 2003; Ouyang et al., 2007; Son et al., 2000).

What are the factors driving age related telomere attrition? Twin studies reveal initial telomere length of peripheral blood lymphocytes to be genetically determined (Slagboom et al., 1994) and its subsequent diminution with age is primarily a consequence of repeated cellular activation, which drives telomerase loss (Akbar and Vukmanovic-Stejic, 2007; Akbar et al., 2004), and proliferation, whose main drivers include antigen load and inflammation (Aviv et al., 2006; Plunkett et al., 2005; Andrews et al., 2009). In addition, the continuous exposure of cells to free radicals is also considered an significant influence on the maintenance of telomeres (Starr et al.,

2008; Epel et al., 2004), which are highly susceptible to oxidative damage (Kawanishi and Oikawa, 2004; von, 2002).

Furthermore, the novel observation that CMV accelerates the erosion of telomeres in live lymphocytes, $CD8^+$ and $CD4^+$ T cells of healthy donors is also reported. There is one previous report of CMV promoting telomere loss but this was limited to $CD8^+$ T cells and involved patients with coronary heart disease (Spyridopoulos et al., 2009). Shortened telomeres have been observed in peripheral lymphocyte populations of individuals infected with other chronic viral infections such as HIV (Bestilny et al., 2000), HCV and HBV (Satra et al., 2005) and multiple autoimmune diseases (Andrews et al., 2009). These data suggest a common mechanism whereby persistent antigen stimulation results in increased T cell clonal turnover and inflammation which augments the rate of telomere shortening.

The importance of adequate clonal lymphocyte expansion in the optimal function of the adaptive immune system and the potential barrier of replicative senescence may provide a mechanism whereby short telomeres may contribute towards age and CMV associated immune dysfunctions. However, cellular telomere length also serves as a biological indicator of both the proliferative history and the replicative capacity of somatic cells (Allsopp et al., 1992; Hodes et al., 2002). Therefore, short telomeres may either be a cause or simply represent a biomarker of immune system ageing. Nevertheless, data supporting the causal hypothesis include a number of genetic diseases characterized by deficient telomere maintenance that are associated with premature ageing, such as DKC (Armanios, 2009) or ataxia telangiectasia (Akbar et

al., 2004) and animal models, as observed in the telomerase deficient mouse (Blasco, 2002; Akbar et al., 2004). Moreover, mice born with short telomeres, even in the presence of normal telomerase, exhibit rapid onset of ageing (Armanios et al., 2009). Furthermore, enhancing telomerase activity in HIV specific $CD8^+$ T cells dramatically restored their antiviral function (Dagarag et al., 2004; Fauce et al., 2008).

KLRG1 and CTLA-4 expression both display inverse correlations with $CD8^+$ T cell telomere length. Nevertheless, they also exhibit significant positive correlations with age (chapter 3), which itself is inversely correlated with $CD8^+$ T cell telomere length. To better define the functional relevance of the inhibitory receptor expression of a T cell on its telomere length, blocking studies were performed. However, neither CTLA-4, PD-1 nor KLRG1 blockade augmented telomerase activity of $CD8^+$ T cells at any stage of their differentiation. Nevertheless, this may reflect these receptors functioning through PI3K/Akt (as described in section 1.5.4.3), whereas the Ras/p38MAPK/Erk pathway may be the major route through which telomerase is regulated (Fauce et al., 2008; Maida et al., 2002). There is a report of PD-L blockade augmenting telomerase activity, as well as mediating proliferative increases, of HIV specific $CD8^+$ T cells (Lichterfeld et al., 2008). However, the authors did not take into account that T cells upregulate telomerase when induced to proliferate and didn't adjust their experiment accordingly, whereas our experiments were normalised to 500 $Ki67^+$ cells per reaction. Indeed, we observed significant increases in Ki67 proliferation following PD-L blockade, suggesting that if we had not standardised the number of $Ki67^+$ cells, we would have similarly observed increased telomerase

activity. The mechanisms driving these age and CMV associated inhibitory receptor variations were further investigated in the next chapter.

6 Determination of factors driving CMV and age-associated inhibitory receptor changes.

6.1 Introduction

In the previous three chapters we have characterised T cell inhibitory receptor expression variances with age and CMV status and defined their role in driving age-associated immune decline. This raises questions as to what mechanisms are driving these expression changes and whether they can be manipulated to modify inhibitory receptor expression, which is explored in this chapter.

Extrinsic factors, such as cytokines, in the serum of the aged are heavily implicated in age related immune decline (as described in section 1.2.3.2.4). The role of such cytokines in driving age and CMV associated inhibitory receptor expression changes were investigated in this chapter. In particular, we have chosen to study IL-6, whose well documented positive correlation with ageing and multiple age related diseases (Maggio et al., 2006; Ershler and Keller, 2000) have led it to be labelled the "gerontologists cytokine" (Ershler, 1993), alongside the closely correlated pro-inflammatory cytokine TNFα (Bruunsgaard et al., 2003). Additionally, naïve $CD4^+$ T cells from the aged exhibit impaired differentiation into Th1 and Th2 subsets but display retained or even enhanced ability to generate Th17 effectors (Huang et al., 2008; Tesar et al., 2009), which are broadly implicated in many autoimmune diseases (Mesquita et al., 2009) and are characterised by production of the pro-inflammatory cytokine IL-17, which will constitute an additional focus of my study (Blaschitz and

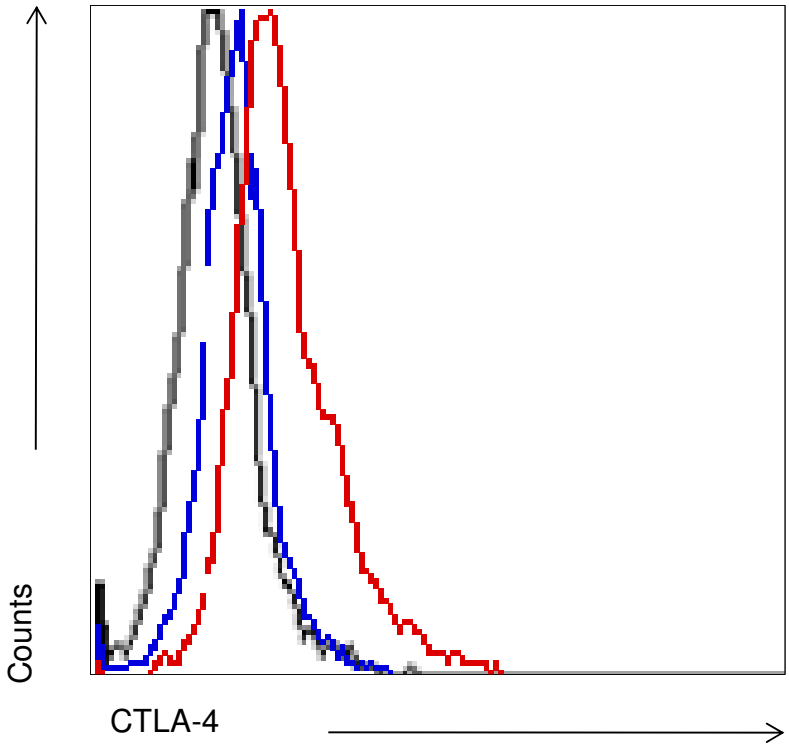
Raffatellu, 2010; van de Veerdonk et al., 2009). Furthermore, we will examine the anti-viral cytokine IFNα, which is secreted at high levels in response to CMV and, by virtue of its ability to induce costimulatory receptor loss and inhibit telomerase activity, has been suggested to be the factor driving accelerated T cell differentiation in CMV infected individuals (Fletcher et al., 2005; Reed et al., 2004). The anti-inflammatory cytokine IL-10, whose activity is critical for the attenuation of immune responses and is exploited by several chronic pathogens to enable their persistence after primary infection (Brockman et al., 2009; Slobedman et al., 2009) was also studied, as any age related immune dysfunction in its production may contribute towards the defective immune responses or pro-inflammatory phenotype of the aged.

The common gamma chain of cytokines were an additional subject of my studies, including IL-2, a key component for the optimal activation and proliferation of B and T cells, whose decreased production is recognised as one of the most consistent age related changes in cytokine production (Pawelec et al., 2001; Gardner and Murasko, 2002). Moreover, multiple studies have revealed that many of the age-associated lymphocyte functional defects can be abrogated following the *in vitro* addition of exogenous IL-2 (as described earlier). Additionally, IL-7 and IL-15 are key regulators in the extrathymic homeostatic proliferation of T cells (Surh and Sprent, 2008). These cytokines have been implicated in driving the increasingly differentiated T cell pool associated with ageing, as IL-15 can induce the loss of CD28 expression on $CD8^+$ T cells (Pawelec, 2007) and loss of IL-7 is correlated with accumulation of T_{EM} $CD4^+$ T cells (Kang et al., 2004). Finally, IL-21 is another common gamma chain cytokine which has been implicated in T cell homeostasis and has been shown to act as an

antagonist to the IL-15 induced loss of CD28 expression on $CD8^+$ T cells (Nguyen and Weng, 2010). IL-21 also is a potent enhancer of NK cell and $CD8^+$ T cell proliferation, IFNγ production and cytotoxicity (Nguyen and Weng, 2010). Thus, age related IL-21 loss may contribute towards the increased differentiation of the $CD8^+$ T cell pool, and the increased tumourigenesis and defective immune responses observed in old age. The effects of these cytokines on $CD4^+$ and $CD8^+$ T cell inhibitor receptor expression were studied alongside the influence of donor age and CMV status, to elucidate the roles of these cytokines in driving age and CMV associated immune alterations.

6.2 Measurement of the effects of cytokines on inhibitory receptor expression

The ability of cytokines to modulate inhibitory receptor expression was measured by anti-CD3 stimulation of PBMCs over a 24 hour period in the presence or absence of the relevant cytokine. The response of $CD4^+$ or $CD8^+$ T cells and the difference in MFI, with and without cytokine, was documented (Representative example, Fig 6.01).



	Stimulus	MFI
———	U/S	5.24
———	αCD3 alone	9.48
———	αCD3 + IL-15	17.1

Figure 6.01 Illustrative example of the quantification of the effects of various cytokines on T cell inhibitory receptor expression.

PBMCs were stimulated with anti-CD3 for 24hrs in the presence and absence of IL-15. Cells were then stained with anti- CD8-PerCP, CD4-FITC and intracellularly with CTLA-4-PE and analysed by flow cytometry. Representative example of CTLA-4 expression, on $CD8^+$ T cells, that were left unstimulated (black line), stimulated with anti-CD3 alone (blue line) and stimulated with anti-CD3 in the presence of IL-15 (red line). The difference in inhibitor receptor expression between αCD3 stimulated PBMC samples in the presence and absence of the cytokine is what was recorded.

6.3 How cytokines modulate the expression of inhibitory receptors

6.3.1 CTLA-4

Only the common gamma chain cytokines IL-2, IL-7, IL-15, IL-21 significantly upregulated CTLA-4 expression on both $CD8^+$ (Fig 6.02A) and $CD4^+$ (Fig 6.02B) T cells, whereas IFNα, IL-6, IL-10, IL-17 and TNFα did not significantly affect CTLA-4 expression. In both $CD8^+$ and $CD4^+$ T cells, IL-15 and IL-2 induced of the greatest magnitude changes in CTLA-4 expression followed by IL-21 and then IL-7.

CTLA-4

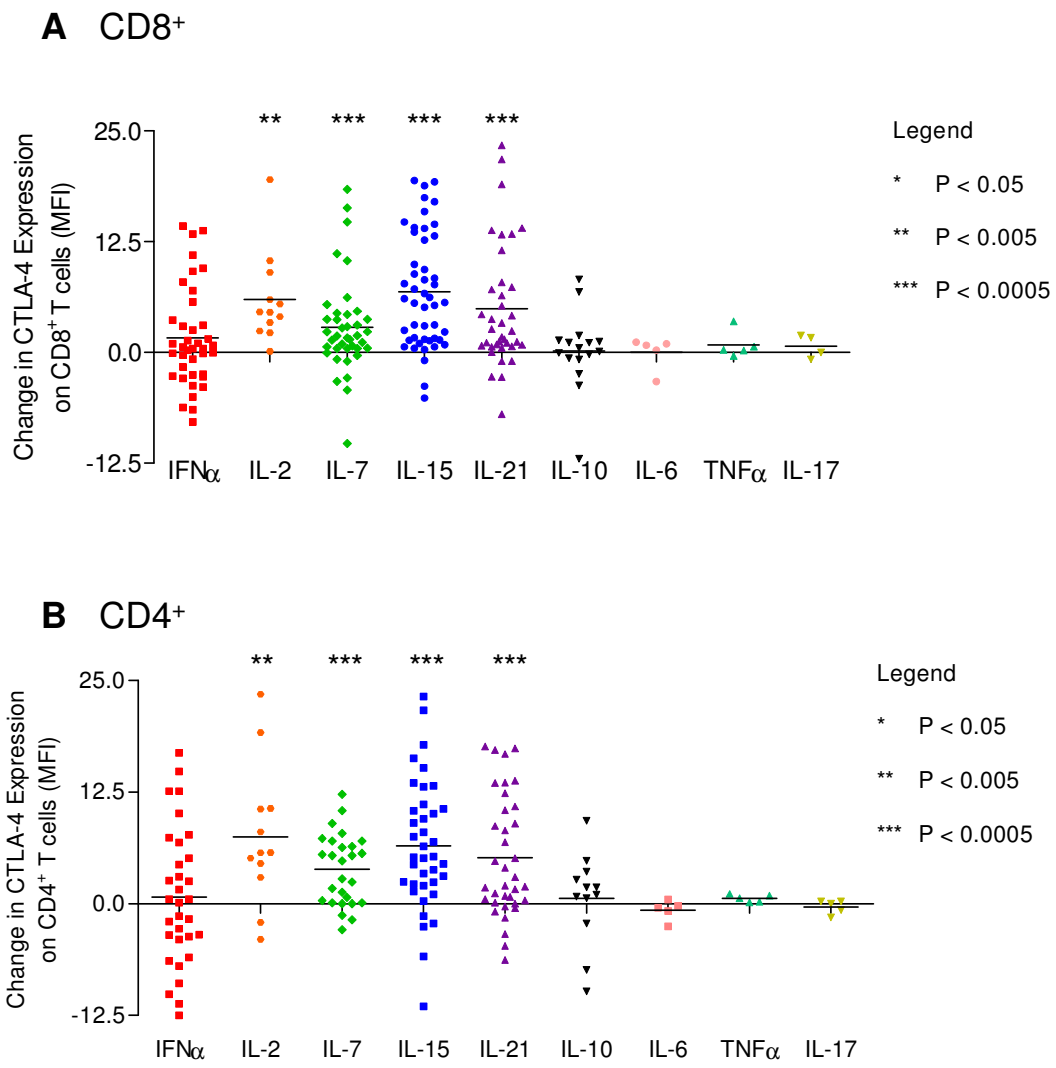


Figure 6.02 The effects of various cytokines on T cell CTLA-4 expression.

The effects of cytokines on CTLA-4 expression was accomplished as detailed in the previous figure. **(A)** Pooled data comparing effects of addition of different cytokines to anti-CD3 induced CTLA-4 expression on $CD8^+$ and **(B)** $CD4^+$ T cells. Horizontal lines represent mean values and P values represent Wilcoxon signed rank tests indicating if data is significantly non zero. Only significant differences are shown.

When comparing the effects of cytokines between young and old donors, it was found that IL-15 and IL-21 were able to upregulate CTLA-4 by greater amounts on old compared to young donors' $CD8^+$ T cells (Fig 6.03A). No age dependent effects of IL-2, IL-7, IL-15 or IL-21 were observed on CTLA-4 expression of $CD4^+$ T cells (Fig 6.03B). Stratifying the effects of cytokines by donor CMV status revealed that CMV^+ donors augmented CTLA-4 production by a greater magnitude than CMV^- ones in their $CD8^+$ T cell response to IL-15 (Fig 6.04A) and on their $CD4^+$ T cells to IL-7 (Fig 6.04B). No other cytokines significantly differed in their effects between CMV^- and CMV^+ subjects.

CTLA-4

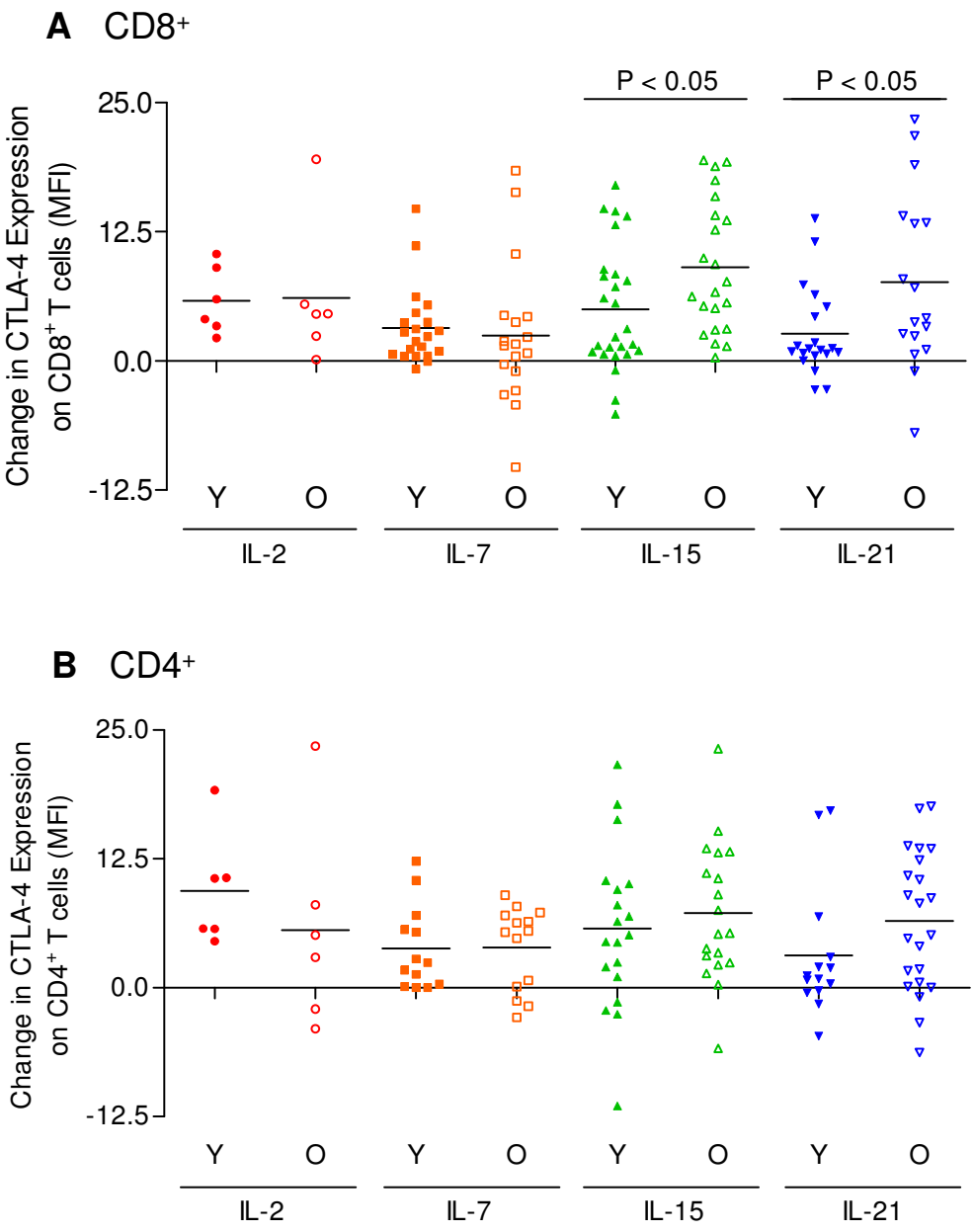


Figure 6.03. Contrasting the effects of cytokines on CTLA-4 expression of T cells in old and young donors.

PBMCs from young and old donors were stained and analysed as described in the figure 6.01. **(A)** Pooled data comparing CTLA-4 expression changes induced on $CD8^+$ and **(B)** $CD4^+$ T cells following coincubation of various cytokines with OKT3 compared with those stimulated in the absence of cytokines in young and old donors. Horizontal lines depict mean values. Filled symbols represent young donors (Y, <35 years) and open symbols represent old donors (O, >65). Statistical significance was assessed using a Mann-Whitney U-test. Significant differences between old and young donors are shown

CTLA-4

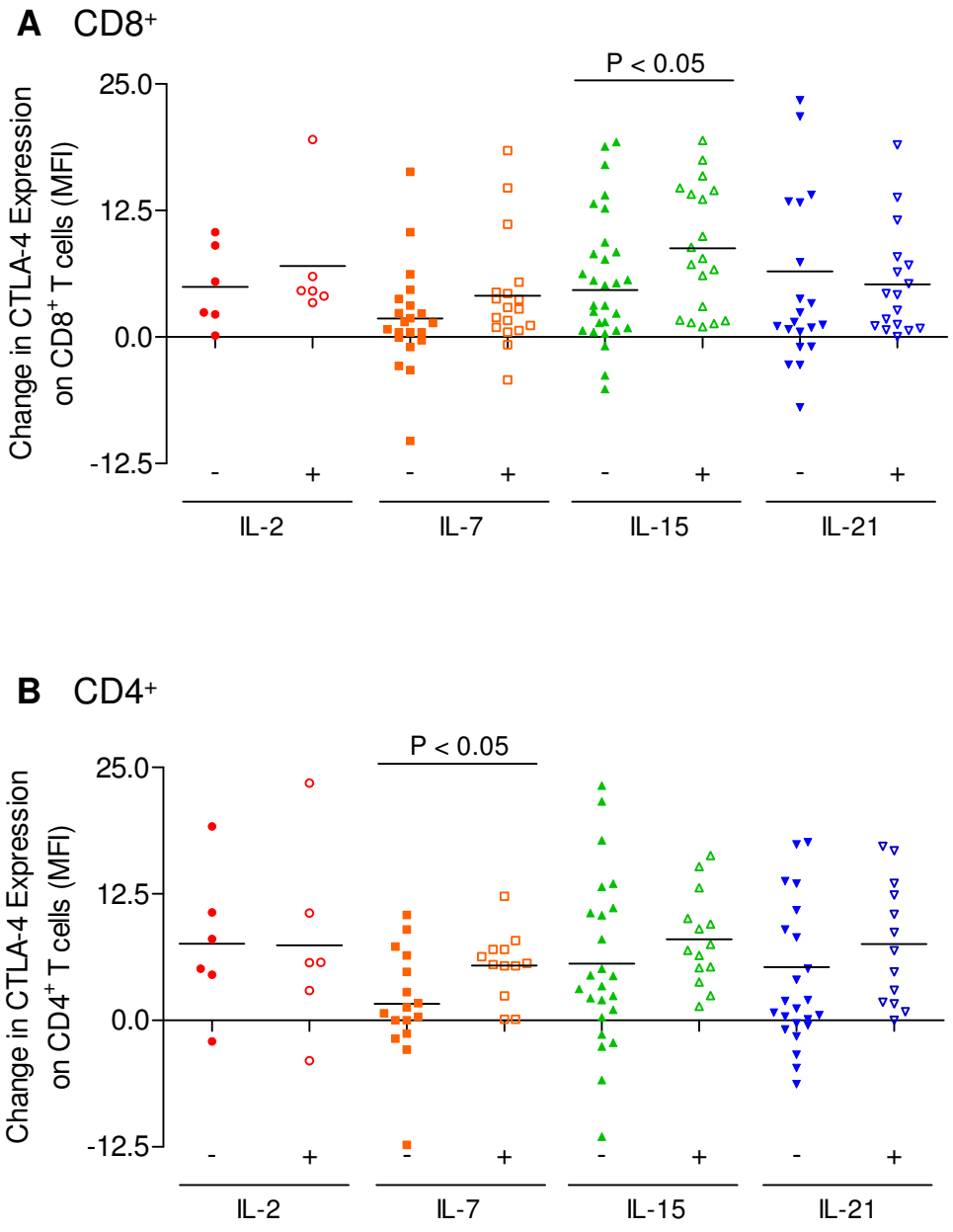


Figure 6.04. Comparison of the effects of cytokines on CTLA-4 expression of CD8⁺ T cells in CMV⁺ and CMV⁻ donors.

PBMCs from CMV⁻ and CMV⁺ donors were treated and stained as detailed in the Fig 6.02. Cumulative data comparing CTLA-4 expression changes on CD4⁺ T cells of CMV⁻ and CMV⁺ donors following IFNα, IL-2, IL-7, IL-15, IL-21, IL-10, IL-6, TNFα and IL-17 co-incubation on **(A)** CD8⁺ and **(B)** CD4⁺ T cells Filled symbols represent CMV⁻ donors and open symbols represent CMV⁺ donors. A Mann-Whitney U-test was used to generate P values and only significant differences between CMV⁺ and CMV⁻ donors are shown

6.3.2 PD-1

Similar to the findings seen using CTLA-4, PD-1 expression on $CD8^+$ and $CD4^+$ T cells was augmented by the common gamma chain cytokines (Fig 6.05). However, unlike CTLA-4, IFNα significantly upregulated PD-1 on both $CD8^+$ and $CD4^+$ T cells and IL-10 significantly diminished PD-1 expression on $CD4^+$ but not $CD8^+$ T cells (Fig 6.05). The magnitude of PD-1 upregulation did not significantly differ between these cytokines. Moreover, stratifying the effects of these cytokines based on donor age did not reveal any significant differences in their responses among the $CD8^+$ or $CD4^+$ T cell compartment (Fig 6.06). Comparing the responses of CMV^- and CMV^+ donors revealed significant differences only in response to IL-7, which induced a significantly higher level of PD-1 expression on the $CD8^+$ T cells of CMV^+ compared with their CMV^- counterparts (Fig 6.07).

6.3.3 KLRG1

No cytokines were able to modulate KLRG1 expression on either $CD4^+$ or $CD8^+$ T cells (data not shown), this is consistent with published data that no known *in vitro* stimulus has been found to upregulate KLRG1 (Voehringer et al., 2002).

PD-1

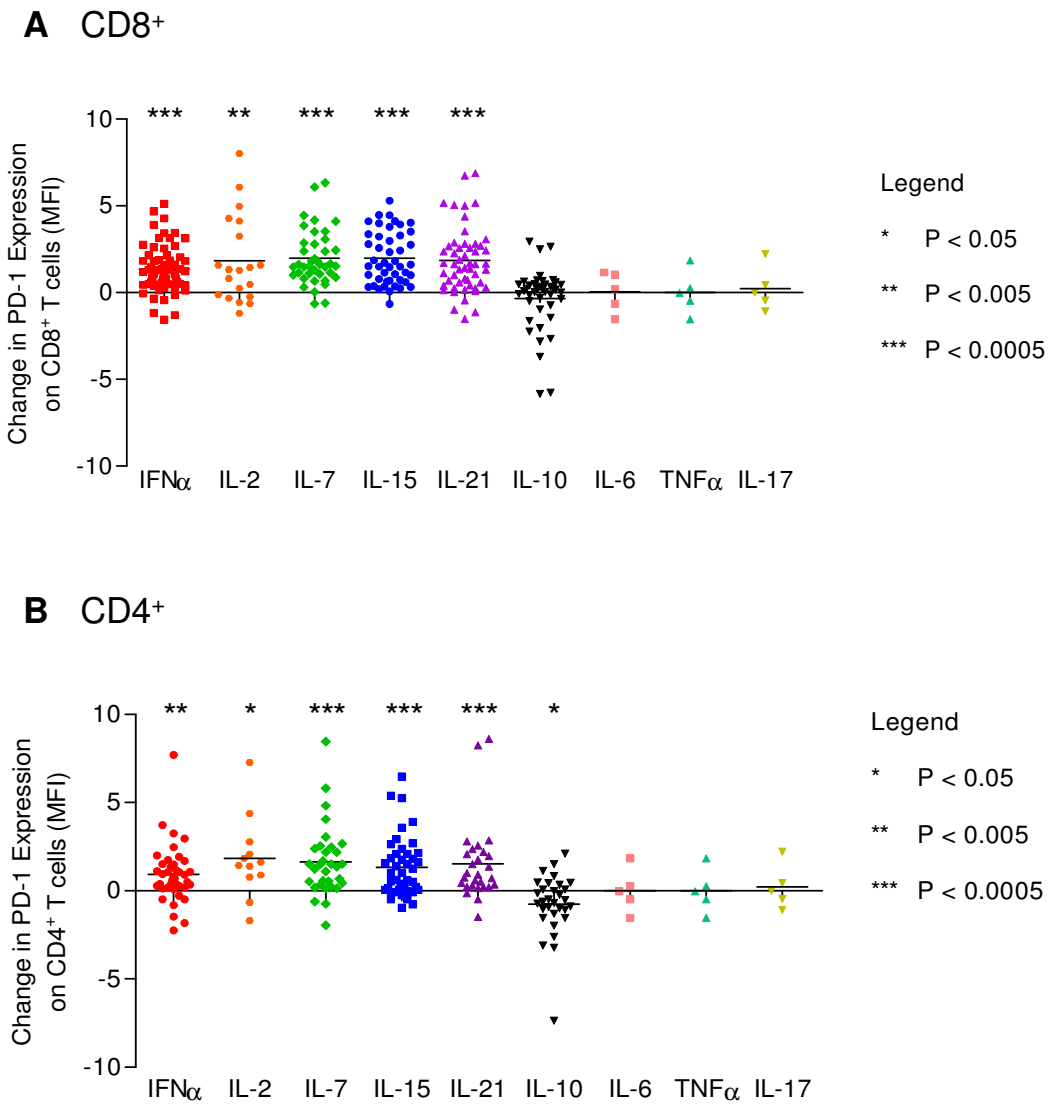


Figure 6.05. PD-1 expression of CD4+ T cells can be altered by various cytokines

PBMCs were stimulated and subject to flow cytomeric analysis as detailed in Fig 6.01 but using anti-CTLA-4-PE rather than an anti-PD-1-PE staining antibody. **(A)** Cumulative data showing PD-1 expression changes on CD8+ T cells following IFNα, IL-2, IL-7, IL-15, IL-21, IL-10, IL-6, TNFα and IL-17 co-incubation on CD8+ and **(B)** CD4+ T cells. Horizontal lines represent mean values and P values represent Wilcoxon signed rank tests indicating if data is significantly non zero. Only significant non zero differences are shown.

PD-1

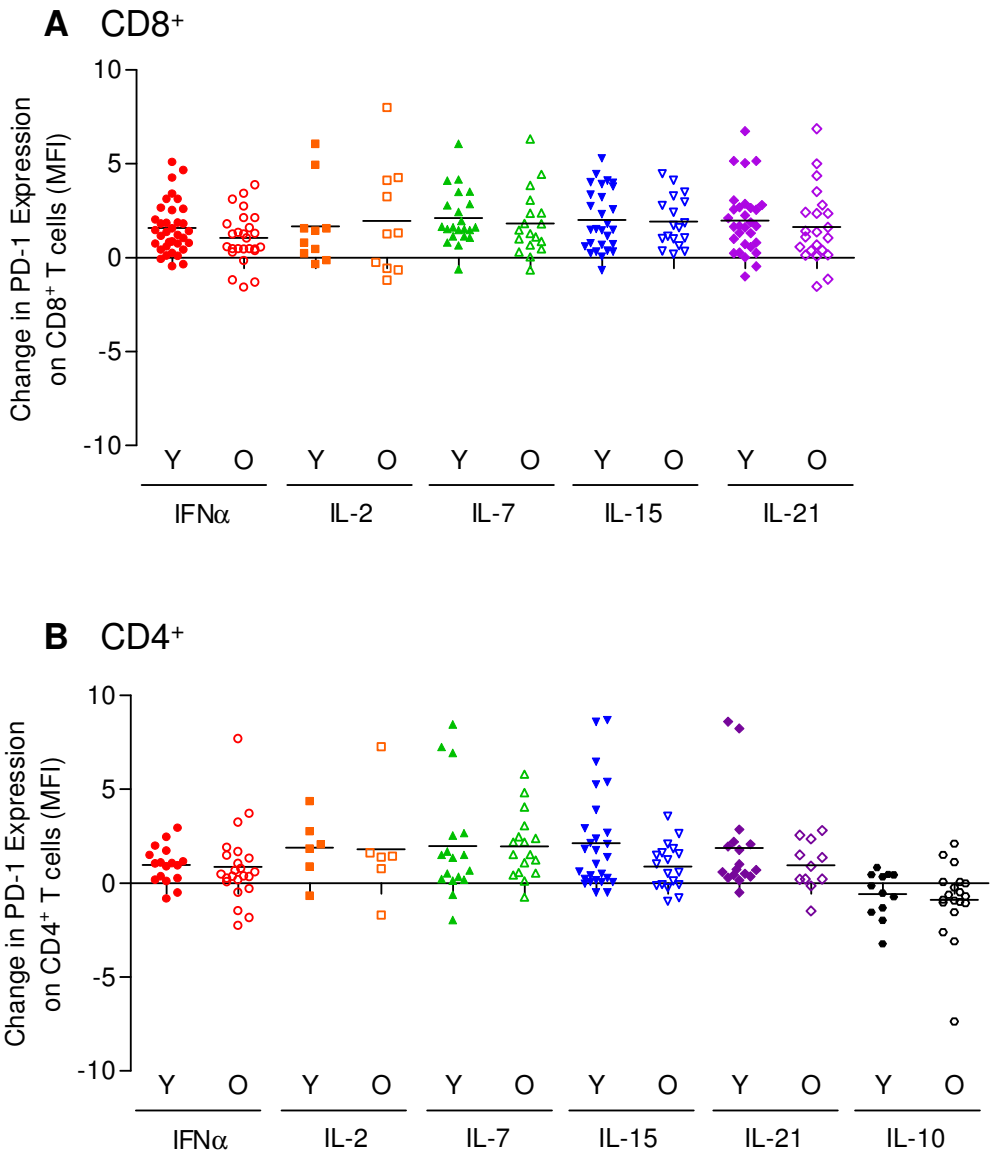


Figure 6.06. The effects of cytokines on PD-1 expression of CD8⁺ and CD4⁺ T cells in old compared with young donors.

PBMCs from old and young donors were stimulated, stained and analysed as depicted in the previous figure. **(A)** Cumulative data showing PD-1 expression changes on OKT3 stimulated CD8⁺ and **(B)** CD4⁺ T cells following cytokine coincubation in old compared to young donors. Filled symbols represent young donors (<35 years) and open symbols represent old donors (>65). Horizontal lines depict mean values. Statistical significance was assessed by a Mann-Whitney U test and only significant differences between old and young donors are shown

PD-1

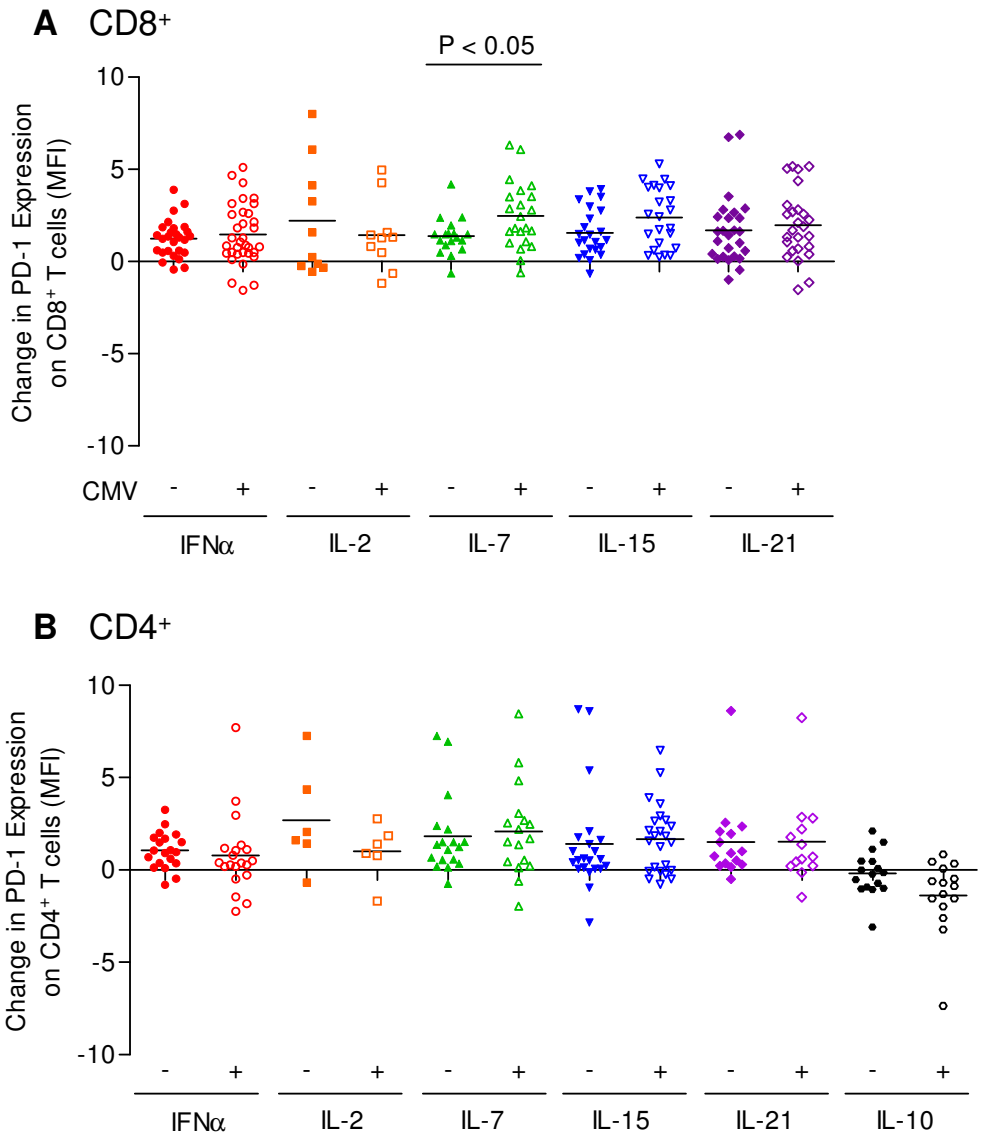


Figure 6.07. Comparing the effects of cytokines on PD-1 expression of T cells based on the CMV sero-status of the donors

PBMCs from CMV$^+$ (open symbols) and CMV$^-$ (filled symbols) donors were stained and analysed as described in Fig 6.05. **(A)** Pooled data comparing differences in PD-1 expression alterations induced by the coincubation of IFNα, IL-2, IL-7, IL-15, IL-21 and IL-10 on αCD3 stimulated CD8$^+$ T cells and **(B)** CD4$^+$ T cells, in CMV$^+$ and CMV$^-$ individuals. Horizontal lines represent mean values. Filled symbols represent young donors (<35 years) and open symbols represent old donors (>65). Statistical significance was calculated using a Mann Whitney U test. Only significant differences between CMV negative and positive donors are shown

6.4 Blocking cytokine receptor signalling can manipulate T cell inhibitory receptor expression levels

Blocking antibodies were used to quantify the significance that these cytokines play in inducing inhibitory receptor expression. PBMCs were stimulated with anti-CD3 in the presence of cytokine receptor blockade or their relevant isotype control and the expression of CTLA-4 and PD-1 on both $CD4^+$ and $CD8^+$ T cells was monitored. The IL-15 cytokine receptor was selected for blocking, as IL-15 induced the greatest CTLA-4 expression changes on both $CD4^+$ and $CD8^+$ T cells. However, IL-15R blockade was unable to significantly alter PD-1 or CTLA-4 expression levels on $CD8^+$ or $CD4^+$ T cells (Fig 6.08). IFNα was also chosen, as it was the only non-common gamma chain receptor that upregulated PD-1 and CMV induces the production of large amounts of IFNα (Fletcher et al., 2005). Blocking IFNα receptors significantly reduced the level of PD-1 expression induced by anti-CD3 by approximately half among both $CD8^+$ (Fig 6.09A) and $CD4^+$ T cells (Fig 6.09B).

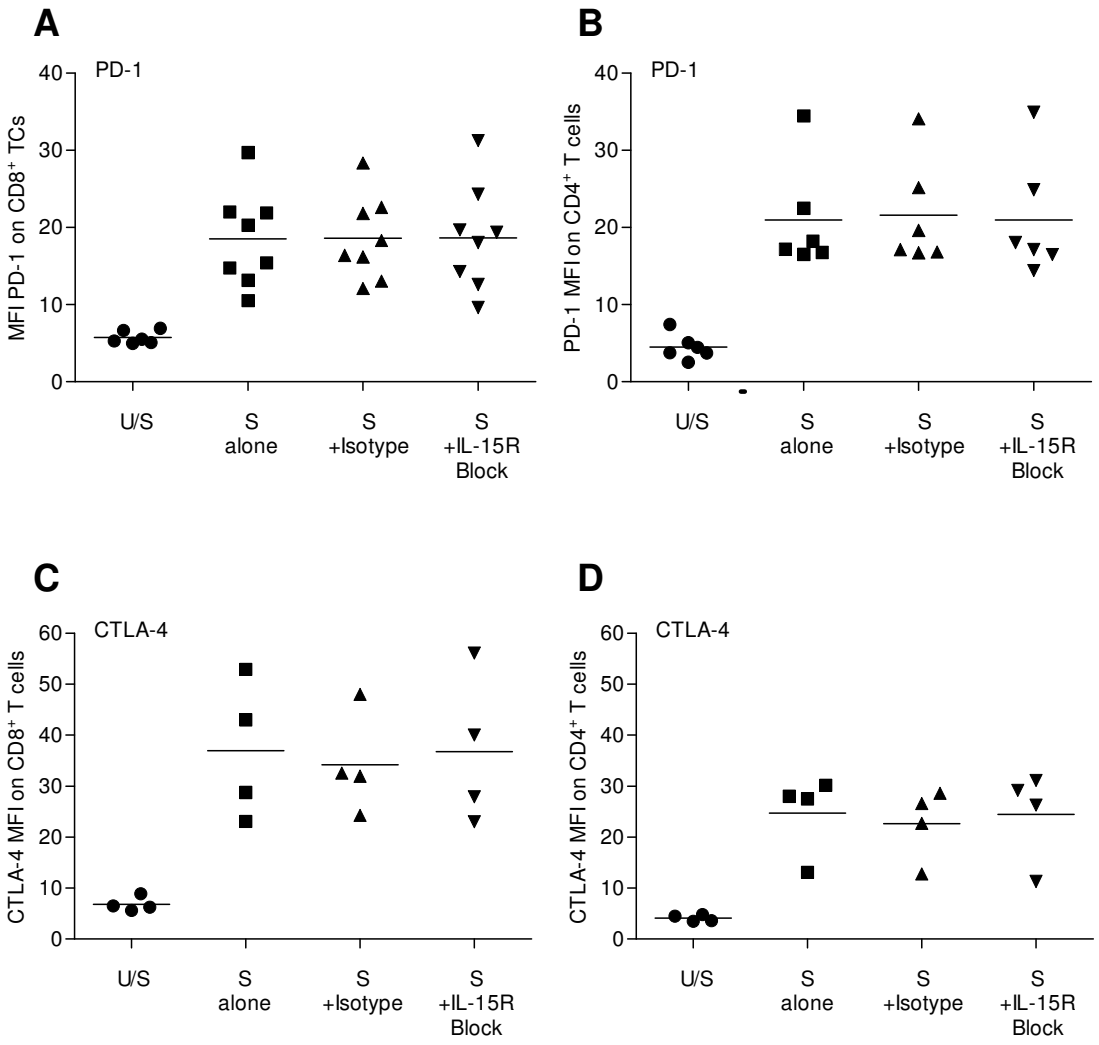


Figure 6.08. The effect of IL-15 receptor blockade on inhibitory receptor expression of CD8⁺ T cells..

PBMCs were stimulated with anti-CD3 for either 48 hours, to measure the effects on PD-1 expression or 24 hours to examine CTLA-4 in the presence of IL-15R blockade or its relevant isotype controls. Cells were then stained using CD4-PerCP, CD8-FITC and either PD-1-PE or intracellularly with CTLA-4-PE. **(A)** Pooled data showing the effect of IL-15R block on the expression of PD-1 on CD8⁺ and **(B)** CD4⁺ T cells. **(C)** Cumulative data illustrating the effect of blocking IL-15R on the expression of anti-CD3 induced CTLA-4 on CD8⁺ and **(D)** CD4⁺ T cells. Horizontal bars depict mean values. The P values were calculated using a Mann Whitney U test. Only differences between inhibitory receptor blockade and its isotype control are shown.

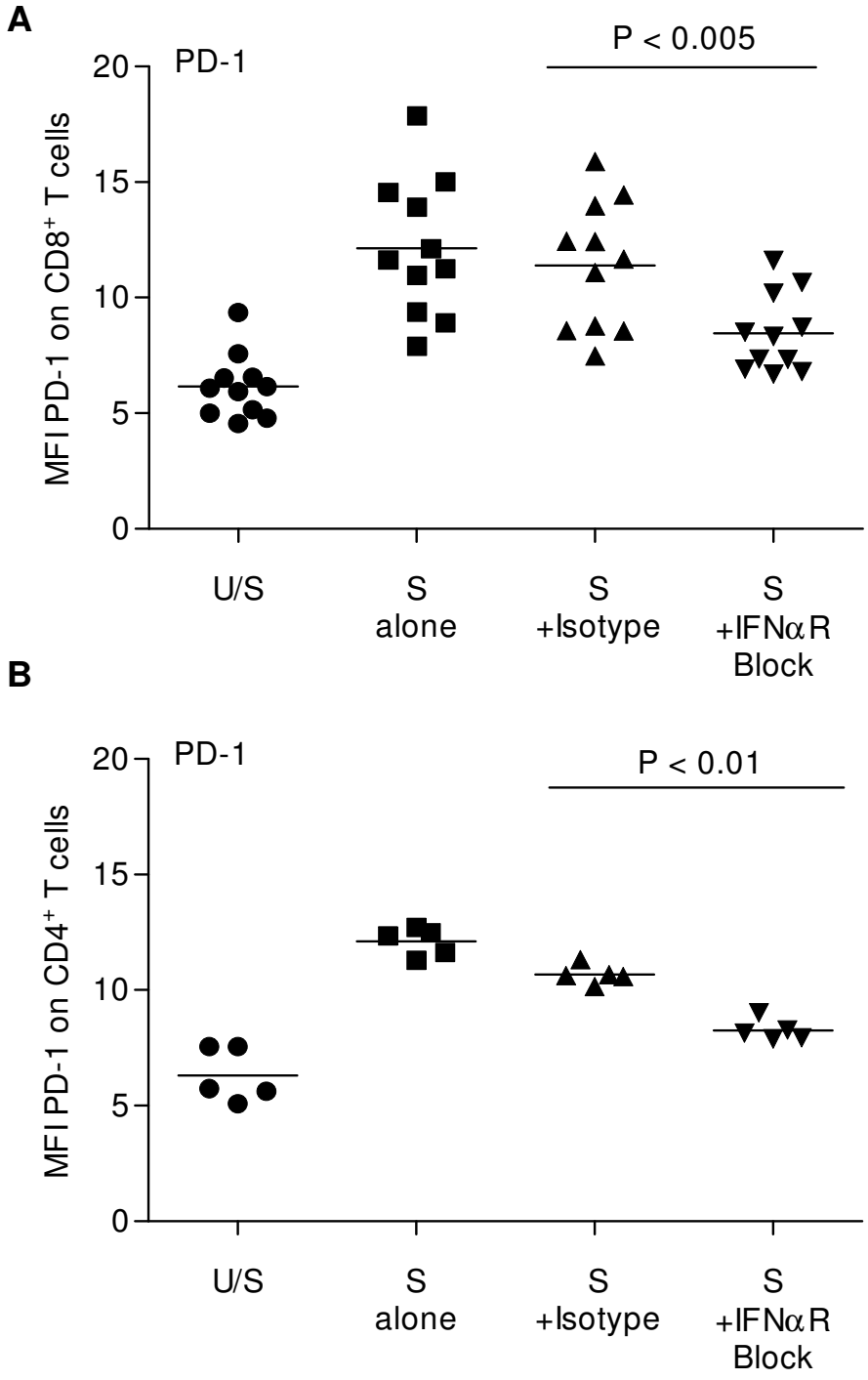


Figure 6.09. Consequences of blocking IFNα receptors on the expression PD-1

PBMCs were stimulated and stained as detailed in the previous figure but using IFNαR blocking antibody rather than an anti-IL-15R block. **(A)** Cumulative data illustrating the effects of IFNαR blockade on the PD-1 expression of $CD8^+$ T cells and **(B)** $CD4^+$ T cells. Horizontal lines indicate mean values. The P values were calculated using a Mann-Whitney U test. Significant differences between receptor blockade and isotype control are shown.

6.5 Discussion

Ageing is characterised by an upregulation of inflammatory markers, however, no pro-inflammatory cytokine tested upregulated CTLA-4, PD-1 or KLRG1 on $CD4^+$ or $CD8^+$ T cells. Indeed, no cytokines of any type significantly augmented KLRG1 expression, consistent with the notion that age and CMV associated KLRG1 expression changes primarily reflect differentiation of the T cell pool. In contrast, the common gamma chain cytokine family members upregulated both CTLA-4 and PD-1 on $CD8^+$ and $CD4^+$ T cells. This ability of homeostatic cytokines to upregulate PD-1 expression was recently confirmed by Kinter *et al. in vitro* and *in vivo*, where it was suggested this may represent a negative feedback mechanism by which homeostatic proliferation is regulated to prevent inappropriate expansion and activation of a single T cell (Kinter et al., 2008). Nevertheless, they were unable to demonstrate that the PD-1 expression induced interfered with homeostatic proliferative expansion and understanding of such a pathway is becoming more important as common gamma chain cytokines are increasingly being used as a therapeutic modality (Sondergaard and Skak, 2009; Leone et al., 2009). Data presented here demonstrate that these cytokines also upregulate CTLA-4, which may represent an additional or alternative mechanism of regulating homeostatic peripheral expansion. Indeed, the large increase in CTLA-4 expression on naïve $CD8^+$ T cells amongst the aged (observed in chapter 3) could reflect a compensatory homeostatic response of these cells undergoing high levels of lymphopoenia-induced expansion as a consequence of age related extreme $CD8^+$ T cell differentiation (Cicin-Sain et al., 2007; Ferrando-Martinez et al., 2010).

Whether common gamma chain cytokines could account for the age and CMV related changes in CTLA-4 and PD-1 expression is unclear, as data suggests that their serum levels either decrease or remain unchanged with increasing age. Nevertheless, classification of cytokine receptors is being increasingly recognised as a more experimentally and clinically useful measure of cytokine activity (Fry and Mackall, 2005), reflecting the pleiomorphic and redundant nature of cytokine responses being a consequence of their homologous receptors. Indeed, IL-15 upregulated CTLA-4 expression on $CD8^+$ T cells by a greater degree amongst aged compared with young donors. This is in accordance with IL-15 receptor expression and cytokine responsiveness increasing as $CD8^+$ T cells differentiate (Geginat et al., 2003). In response to IL-15 and IL-21, old donors exhibited increased CTLA-4 induction on their $CD8^+$, but not $CD4^+$ T cells, compared with their young counterparts, which may account for the association of CTLA-4 expression with age observed only on $CD8^+$ T cells. Similarly, in response to IL-7 and IL-15, CMV^+ donors upregulate significantly more CTLA-4 on their $CD4^+$ and $CD8^+$ T cells, respectively, which may account for the augmented CTLA-4 expression among CMV^+ donors' T cells.

Unlike CTLA-4, the cytokine induced upregulation of PD-1 expression does not significantly differ with donor CMV status. This may reflect expression changes of smaller magnitude being induced compared with CTLA-4, making significant differences between cohorts more difficult to elucidate. Additionally, cryopreserved samples were utilised to compare the upregulation of inhibitory receptors in response to cytokines by donor age and CMV status and recently the cryopreservation process has been associated with reductions in PD-1 expression (Campbell et al., 2009), such

that a correlation established with fresh PBMCs was not observable using cryopreserved samples (Holm et al., 2008). Alternatively, data presented here showing IFNα upregulates PD-1, may account for the T cell pool of CMV^+ donors expressing enhanced levels of PD-1 compared with their CMV^- counterparts. Indeed, large amounts of IFNα are induced as part of anti-CMV immune responses and blocking studies revealed that IFNα alone contributes half the PD-1 expression induced by an anti-CD3 stimulus, highlighting its functional significance. Similarly, the upregulation of PD-1 on the global $CD8^+$ and $CD4^+$ T cell pool of HIV (Trautmann et al., 2006; Day et al., 2006; D'Souza et al., 2007) and the $CD8^+$ T cell compartment of acute HCV infected individuals (Nakamoto et al., 2008) may be mediated through IFNα. Moreover, the high levels of PD-1 expression and comparative absence of CTLA-4 expression on CMV and HIV specific $CD8^+$ T cells (Kaufmann et al., 2007) may reflect IFNα inducing PD-1 but not CTLA-4 expression. Furthermore, IFNα drives T cell differentiation and may significantly contribute towards the CMV and HIV associated global differentiation of the T cell pool (Appay et al., 2007; van de Berg et al., 2008).

The observed differences in age and CMV related cytokine induced inhibitory receptor upregulation may reflect changes in cytokine signal transduction. Indeed, lipid rafts have been suggested to be critical for cytokine signalling as part of the 'raft-STAT' hypothesis (Sehgal et al., 2002). Thus, the compromised function of lipid rafts amongst the increasingly rigid cell membrane of the aged, as described in section 1.2.3.2.5, predicts a generalised defect in cytokine signalling amongst old donors. Nevertheless, my data illustrates the ability of common gamma chain receptor

cytokines to upregulate CTLA-4 and PD-1 is generally maintained and in a few cases actually enhanced amongst the aged. Little data exists on age related signalling alterations of common gamma receptor cytokines with age but this finding is consistent with old donor T cells exhibiting no age related deficit in IL-7R signalling (Nasi et al., 2006; Kang et al., 2004). Enhanced cytokine signal transduction may also reflect age related exhaustion of negative regulators that inhibit cytokines responses, such as SHP-1 and SHP-2 phosphatases, protein inhibitors of activated stats (PIAS) and suppressors of cytokine signalling (SOCS), as suggested by (Fulop et al., 2006).

Nevertheless, other explanations for inhibitory receptor expression variances amongst different cohorts, independent of cytokines, exist. For example, CTLA-4 and PD-1 are internalised following surface expression (Alegre et al., 1996; Pentcheva-Hoang et al., 2007; Raimondi et al., 2006) and defective internalisation has been suggested to underlie the age related increase in inhibitory receptor expression in mice (Shimada et al., 2009).

7 Overall discussion

The fate of cellular immune responses is critically dependent on the balance between signals delivered by positive and negative costimulatory receptors. Manipulation of T cell inhibitory receptor pathways constitutes a novel approach for re-invigorating exhausted T cell responses in the context of chronic infections. T cells also exhibit profound exhaustion in old age and this thesis investigates the hypothesis that the modulation of T cell inhibitory receptors may have the potential to reverse the changes seen in age onset immunodecline.

7.1 Reinvigorating CMV specific $CD8^+$ T cell function by manipulation of the PD-1/L axis

The PD-1/L pathway is revealed to define a reversible defect among CMV specific $CD8^+$ T cell proliferative responses. More specifically, CD45RA-revertant memory CMV specific $CD8^+$ T cells exhibit a proliferative deficit compared with central and effector memory T cells, which is reversed following PD-L blockade, an effect demonstrated to be independent of T cell phenotypic changes. Overall, these data demonstrate that PD-1 plays a role in the dysregulation of $CD8^+$ T cell responses to CMV, which constitutes a major force driving T cell immunosenescence (see section 1.3.2.2). Moreover, reducing the chronic antigen burden may impair the development of immunosenescence (Kassu et al., 2003; Kalinkovich et al., 1998; Romanyukha and Yashin, 2003). Furthermore, despite critical roles of PD-1 in the development and maintenance of tolerance (as described in section 1.5.3.2.2), its blockade *in vivo* is

remarkably well tolerated (Velu et al., 2009; Berger et al., 2008). Therefore, manipulation of the PD-1/L axis may partially reinvigorate CMV specific $CD8^+$ T cell function and contribute to improved control of CMV reactivation, reducing its effects in driving extreme T cell differentiation and the accumulation of oligoclonal expansions.

The failure, however, of PD-L blockade to augment telomerase expression in virus specific $CD8^+$ T cells would suggest that, rather than representing true functional reversal of exhaustion, blocking PD-L drives a short term functional increase that will further exhaust these already near senescent cells. Moreover, my studies solely investigated the peripheral blood compartment, however virus specific and bulk $CD8^+$ T cells residing in peripheral sites of viral replication are more differentiated and exhausted than those from the circulating pool (van Leeuwen et al., 2006a; Blackburn et al., 2010) and may be refractory to PD-1/L blockade (Nakamoto et al., 2008). Furthermore, pp65 restricted HLA-A2 or -B7 tetramers were used to study CMV specific $CD8^+$ T cell function, as it has been well documented that the CMV specific $CD8^+$ T cell responses are extremely focussed on just two epitopes: IE-1 and pp65 (Kern et al., 1999; Khan et al., 2007; Wills et al., 1996). However, many other viral proteins can encode immunogenic epitopes and $CD8^+$ T cell recognition of CMV is unusually broad, complex and poorly approximated by responses to any one or two open reading frames (ORFs) (Lilleri et al., 2009; Sylwester et al., 2005). Indeed, although the majority of studies on CMV specific $CD8^+$ T cells have focussed on IE-1 and pp65, it can not necessarily be assumed that the largest detectable response corresponds as the most critical for the viral control. Thus, the behaviour of pp65

specific $CD8^+$ T cells in response to PD-L blockade may not reflect the behaviour of those cells specific for the precise antigens crucial for the control of CMV.

In vivo studies, nevertheless, reveal that PD-1/L blockade results in a $CD8^+$ T cell response of much higher quality, resulting in better control of LCMV (Barber et al., 2006), SIV (Velu et al., 2009) and cancer (Berger et al., 2008). This may reflect that *in vivo* PD-1 blockade may not reinvigorate exhausted cells but instead may function during the priming phase to prevent $CD8^+$ T cells from becoming exhausted, as demonstrated cancer and infections of MCMV and Friend virus (Matsuzaki et al., 2010; Benedict et al., 2008; Takamura et al., 2010). PD-L blockade may also abrogate the PD-L1 mediated induction of iTregs, whose numbers are augmented in chronic infections (Belkaid, 2008).

7.2 Other immuno-rejuvenation strategies

Aside from inhibitory receptor blockade to reinvigorate exhausted CMV specific $CD8^+$ T cell functions, several alternative immuno-rejuvenation strategies are also being explored. Indeed, rather than improving their function, depletion of the exhausted CMV specific $CD8^+$ T cell clones could allow a smaller number of less differentiated, more functional cells to replace them, (Touvrey et al., 2009; Weinberger et al., 2009) freeing up immune space for other naïve and memory T cells (Alexander et al., 2009). Alternatively, eradication of CMV has been suggested to be a desirable goal and indeed, a partially effective prophylactic vaccine has recently been developed (Pass et al., 2009). However, a few beneficial effects of CMV

infection have been documented, including possibly being protective against infectious disease (Barton et al., 2007), cancer (Pawelec et al., 2010a) and transplant rejection (Nickel et al., 2009; Pawelec and Derhovanessian, 2010), so there may be consequences resulting from its elimination. Other possible interventions could include thymic rejuvenation strategies (reviewed in (Pinti et al., 2010)), long term treatment with anti-inflammatory and/or anti-viral agents (Pawelec and Derhovanessian, 2010) and stress reducing behavioural therapies (Irwin et al., 2007; Epel et al., 2009; Gouin et al., 2008).

7.3 Roles of CTLA-4 in age onset immune decline

Although CTLA-4 expression increased on $CD8^+$ T cells with age, its blockade did not reverse the characteristic hypo-responsiveness of neither CMV specific nor aged $CD8^+$ T cells, suggesting that CTLA-4 may not play a direct role in age onset immune decline. This is at odds with the observed CTLA-4 upregulation with both CMV sero-positivity and age on $CD8^+$ T cells. Nevertheless, SLE patients exhibit augmented CTLA-4 expression on their $FOXP3^-$ T cells but this CTLA-4 exhibits an impaired ability to regulate T cell signalling, as it is excluded from lipid microdomains (Jury et al., 2010). Significant alterations to the lipid content of cell membranes of aged humans have also been documented (as described in section 1.2.3.2.5) that could compromise CTLA-4 signalling, despite its increased expression. Alternatively, this may reflect the fact that the blocking antibodies utilised may not have been physically capable of accessing an immune synapse due to their large size, which may be particularly relevant to CTLA-4, a predominantly intracellular molecule that is

expressed directly into the immune synapse (as described in 1.5.3.1). Indeed, different preparations of anti-CTLA-4 antibodies have different effects on cellular functions (Sansom and Walker, 2006) and the best blockers in terms of affinity and ligand binding perturbation did not have the most potent CTLA-4 modulatory effects (Sansom and Walker, 2006; Chandraker et al., 2005). Alternatively, upregulation of CTLA-4 amongst old and CMV^+ donors also mirrors CMV and age-associated downregulation of the co-stimulatory molecule CD28 (Boucher et al., 1998; Looney et al., 1999; Czesnikiewicz-Guzik et al., 2008). Indeed, when taken together with a report indicating that CTLA-4 may deliver its inhibitory function via promoting the internalisation and degradation of CD28 molecules (Berg and Zavazava, 2008), the age and CMV associated CD28 expression loss may be consequence of enhanced CTLA-4 expression. Alternatively, CTLA-4 can also exert dominant suppression via its ligands into APCs (Munn et al., 2002), thus its augmented expression may significantly contribute towards the impaired immune responsiveness of old age.

7.4 Functions of KLRG1 in immunosenescence

KLRG1 expression increases with age on T cells and is highly expressed on CMV specific $CD8^+$ T cells and perturbation of its signalling significantly increased the proliferative activity of highly differentiated $CD8^+$ T cells from young but not old donors. In addition, KLRG1 signalling blockade also failed to augment the proliferative $CD8^+$ T cell responses of aged donors to levels observed in the young, nor enhance CMV specific responses. This suggests that although KLRG1 signalling is causative of dysfunction in highly differentiated $CD8^+$ T cells of the young, the

functional exhaustion experienced by chronic virus specific and old $CD8^+$ T cells involve additional mechanisms, possibly including induction of distinct inhibitory receptors and the increasing cholesterol content and rigidity of cell membranes of the aged (as described in section 1.2.3.2.5), independent of KLRG1 signalling. However, E-cadherin blocking antibodies were used to pertubate KLRG1 signalling but E-cadherin is expressed on multiple leukocyte subsets, particularly myeloid DCs (Henson et al., 2009) and is being increasingly implicated in the mediation of critical inhibitory signals (Banh et al., 2009; Fu and Jiang, 2010), thus the increased functional responses following E-cadherin blockade may reflect activation of DCs rather than suppression of KLRG1 signalling. Nevertheless, it should be noted that the potential of KLRG1 blocking antibodies to cross-link and provide a negative signal mean that E-cadherin blocking antibodies were the best available reagent. Data presented here also illustrate upregulation of KLRG1 on T cells with age, CMV infection and differentiation state, higher levels of expression on $CD8^+$ than $CD4^+$ T cells and augmented expression on CMV specific $CD8^+$ T cells, which further increases with age. These data support the idea that increased cellular replication histories; associated with chronic infection, increasing age and differentiation state, drive T cell KLRG1 expression (Thimme et al., 2005; Voehringer et al., 2002).

7.5 Other candidate inhibitory receptors involved in CMV specific $CD8^+$ T cell dysregulation

Data presented here depict that PD-1 is not universally expressed on CMV specific $CD8^+$ T cells and its blockade does not fully reinvigorate their functions. This is

consistent with data from other viral infections, both in terms of PD-1 expression (Wang et al., 2007; Day et al., 2006) and incomplete functional restoration by PD-1/L1 blockade (Barber et al., 2006) suggesting the involvement of other molecules. However, since I began my PhD an increasingly diverse array of inhibitory molecules have been implicated in regulating T cell exhaustion, with gene expression profiles revealing exhausted $CD8^+$ T cells co-expressing up to seven inhibitory receptors (Wherry et al., 2007; Blackburn et al., 2009). Indeed, one of these inhibitory receptors, Tim-3, is expressed on a distinct subpopulation of HIV specific $CD8^+$ T cells to PD-1 and has been shown to enhance their functional responses when blocked (Jones et al., 2008). Moreover, $CD8^+$ T cells specific for LCMV, HCV and tumours co-express Tim-3 and/or the inhibitory receptor LAG-3, alongside PD-1, and their blockade can synergise with blocking PD-L in reinvigorating the functions of these cells (as detailed in section 4.1). In summary, the expression of multiple inhibitory receptors by exhausted $CD8^+$ T cells may represent a multitude of redundant pathways that control immuno-pathology in a variety of different circumstances, determined by their different ligand distributions. These receptors may also have non-overlapping intracellular targets enabling each to mediate a distinct inhibition of different cellular functions that together produce an exhausted phenotype. Nevertheless, the exhaustion mediated by inhibitory receptor signalling does not account for all the dysfunctions of CMV specific $CD8^+$ T cells, with senescence also making a significant contribution. Indeed, inhibition of the key senescence signalling protein p38 augments CMV specific $CD8^+$ T cell function and has an additive effect in combination with PD-L blockade (Henson et al, in preparation).

7.6 Reasons for the putatively unique effects of CMV in age related immune decline, compared with other pathogens

Data presented here further illustrate the dominant role of CMV infection in driving age-associated T cell changes in causing dramatic phenotypic and inhibitory receptor expression changes, dominating the immune response with age and accelerating telomere attrition. Reasons for this unique effect of CMV compared with other pathogens have not been fully elucidated but it may reflect the unique replication pattern of CMV. Indeed, latent infections such as herpes simplex virus (HSV), Epstein Barr Virus (EBV) and Varicella zoster virus (VZV) are initially cleared and subsequently undergo a well-defined transcriptionally near silent latency with periodic reactivations (Brunner et al., 2010), whereas persistent viruses like HCV and HIV are never cleared and maintain high viraemia (Wherry and Ahmed, 2004). In contrast, CMV is often characterized as a "smouldering" chronic infection lying in between the two ends of this spectrum (Wherry and Ahmed, 2004), which may reflect three unusual features of CMV: that antigen clearance after primary infection can require over a year (Campbell et al., 2008), its very high frequency of reactivations (McVoy and Adler, 1989; Wiesel et al., 2009; Kurz and Reddehase, 1999) and having a much less well defined latency which may not be transcriptionally silent (Kurz and Reddehase, 1999) and may even undergo a continuous low level productive chronic infection of salivary gland and kidney epithelial cells (Brunner et al., 2010). Alternatively, the unique role of CMV may reflect the cell types it infects: endothelial, monocytes/macrophages and DCs, with their roles as critical mediators of immune responses, body wide distribution and frequent reactivation associated with

inflammation, enabling CMV to exert a strong and persistent systemic immune stimulation. Additional factors underlying these effects of CMV infection may include its ability to activate DC secretion of the differentiation inducing cytokines: IFNα (Fletcher et al., 2005) and TNFα (Geist et al., 1997; Bryl et al., 2005), and it may also act to reduce thymic output, possibly by infecting bone marrow progenitor cells (Jimenez et al., 2006).

7.7 How CMV drives age related immunological changes

In old people, the effects of extended periods of persistent CMV infection may reach a climax with the development of the Immune Risk Phenotype (Pawelec et al., 2009a). Indeed, data presented here illustrate that CMV infection accelerates the rate of age-associated lymphocyte telomere erosion, implicating duration of infection as a critical factor underlying CMV associated immune decline. Alternatively, such data may reflect exacerbation of the effects of CMV by the thymically insufficient, subclinical inflammatory and immuno-depressive environment of the aged, which may increase the frequency (Stowe et al., 2007) and exacerbate the consequences of CMV reactivation (Sauce et al., 2009; Peggs et al., 2003). Nevertheless, phenotypic alterations have been demonstrated to occur very rapidly upon primary CMV infection, amongst both healthy and immunocompromised donors (Wills et al., 2002; Khan et al., 2007; van de Berg et al., 2008; van de Berg et al., 2010; van Leeuwen et al., 2006a; Waller et al., 2008; Elbou Ould et al., 2004). Data presented here illustrate that CMV seropositivity results in phenotypic and immune inhibitory receptor changes of similar magnitude amongst both old and young populations, suggesting

that such differences are not caused by duration of CMV infection. Thus, the role of CMV infection in driving age-associated changes may reflect global changes to the host T cell profile occurring rapidly upon primary infection and the subsequent effects of continuous lifelong immune surveillance driving accelerated T cell senescence.

7.8 CMV infection: a cause or an effect of immunosenescence?

Data presented in this thesis adds to the growing body of literature that implicates CMV infection in the acceleration of age-associated immune decline. Nevertheless, rather than a contributory factor towards the development of immunosenescence, CMV sero-positivity may function as a mere marker of immune competency. Indeed, there is a strong independent relationship between both socioeconomic status (SES) and race with health outcomes, whereby caucasians and those of greater socioeconomic privilege tend to enjoy better health and greater longevity (Farmer and Ferraro, 2005). An analysis of The National Health and Nutrition Examination Survey (NHANES) IIIs data, involving several thousand American individuals, reveals significant racial and socioeconomic disparities in CMV seroprevalence beginning at early ages and persisting into middle age, with lowest rates of infection observed amongst white racial groups and higher income groups (Dowd et al., 2009). Moreover, this study accounted for a wide range of exposures commonly cited as risk factors for CMV and found that differences in these potential exposures did not easily explain the relationship between race, SES and CMV status. Furthermore, individuals from long lived families appear to be more resistant to CMV infection and even after infection are less susceptible to CMV associated immune changes (Pawelec et al.,

2009b; Derhovanessian et al., 2010) and there is also data suggesting that individuals experiencing an increased level of stress, which is associated with lower SES, undergo more frequent CMV reactivation (Esterling et al., 1993; Glaser and Kiecolt-Glaser, 1997; Glaser et al., 1999; Mehta et al., 2000; Herbert and Cohen, 1993). Possibly most significantly, the Leiden Longevity Study has recently disputed the association of CMV with an Immune Risk Phenotype and premature mortality (Maier et al, unpublished observations). In summary, rather than CMV driving many of the deleterious immune changes commonly cited as age-associated, there is evidence that individuals who are more susceptible to these age related immune changes are also more likely to become infected with CMV and that such individuals, once infected are likely to experience more frequent CMV reactivation.

7.9 Further Work

Extending my studies from independent community dwelling older adults to include those institutionalised in hospitals or care homes would help clarify if the correlations observed with ageing are contributory towards the ageing process or if they reflect adaptations to successful ageing.

The effects of PD-1/L blockade on functions of CMV specific $CD8^+$ T cells other than proliferation and telomerase, such as cytokine production, cytotoxicity and apoptosis, will be interesting to quantify. Additionally, extending these studies to include different non-pp65 CMV epitopes, would allow the investigation of whether the

effects on pp65 specific $CD8^+$ T cells are representative of the anti-CMV $CD8^+$ T cell response.

Additionally, it will be important to characterise the expression and functional relevance of additional inhibitory receptors such as LAG-3, Tim-3, CD160, PTGER4 and LILRB (Blackburn et al., 2009; Wherry et al., 2007) to determine whether additional blocks could act in synergy with PD-1 to fully reverse the exhaustion of CD45RA-revertant memory and CMV specific $CD4^+$ and $CD8^+$ T cells.

The utilisation of siRNA techniques to specifically knock out inhibitory receptor genes would represent a more accurate quantification of the role of inhibitory receptors than using inhibitory receptor blocking antibodies, as it would account for the inability of blocking antibodies to access the immune synapse.

Furthermore, the potential use of manipulating inhibitory receptor signalling as a therapeutic modality in cancer and chronic infections would be aided by clarifying whether PD-1/L blockade functions *in vivo* through reinvigorating exhausted $CD8^+$ T cells, preventing naïve T cells developing an exhausted phenotype or inhibiting the PD-1/L mediated induction of iTregs.

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